Tesis Doctoral

Sistemas sexuales y polimorfismo de color en *Silene*: una aproximación en la sección *Psammophilae*

Inés Casimiro-Soriguer Camacho

Sevilla 2015

Dpto. Biología Molecular
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Memoria presentada por la licenciada en Biología **Inés Casimiro-Soriguer Camacho** para optar al título de Doctora en Biología (Doctorado Internacional) por la Universidad Pablo de Olavide de Sevilla.

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Directores

Dr. Maria Luisa Buide del Real

Dr. Eduardo Narbona Fernández

Dr. Montserrat Arista Palmero

A mis padres

A mi hermano

A Carlos

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ÍNDICE

RESUMEN

Silene L. (Caryophyllaceae) presenta una gran variabilidad de caracteres morfológicos y ecológicos. En concreto, la diversidad de sistemas sexuales y su evolución, así como las variaciones de color floral, son posiblemente los aspectos de mayor interés. En esta tesis doctoral realizamos una revisión de las principales estrategias reproductivas en *Silene* así como una estima de la frecuencia de los diferentes sistemas sexuales a nivel de género y subgénero. Posteriormente elegimos la sección *Psammophilae* (Talavera) Greuter (endémica de la Península Ibérica e Islas Baleares) como grupo de estudio para analizar el dimorfismo sexual, así como la frecuencia, mantenimiento y posibles ventajas reproductivas del sistema sexual ginodioico-ginomonoico. Dentro de este grupo, nos centramos en *S. littorea* Brot. para estudiar más detalladamente las variaciones en la expresión sexual durante todo el período de floración. Finalmente, estudiamos la frecuencia, así como las bases bioquímicas y genéticas del polimorfismo de color de los pétalos (rosa fuerte, rosa pálido y blanco) en *S. littorea*.

La revisión de los sistemas sexuales en *Silene* mostró que el hermafroditismo es el sistema sexual más frecuente (58%), seguido de la dioecia (14%), ginodioecia (13%) y ginodioecia-ginomonoecia (12%). Estos sistemas sexuales están representados en los dos subgéneros, *Silene* y *Behenantha* (Otth) Endl. que constituyen dos clados distintos, lo que indica que posiblemente han surgido de forma independiente. La mayoría de poblaciones de las especies de la sección *Psammophilae* presentaron ginodioecia-ginomonoecia, siendo el morfotipo hermafrodita el más frecuente y el porcentaje de plantas femeninas muy bajo en todas las poblaciones. El estudio del dimorfismo sexual y sus consecuencias evolutivas reveló que las flores hermafroditas fueron más grandes, produjeron más óvulos y tuvieron mayores cargas polínicas que las femeninas. Además,

los gradientes de selección mostraron selección direccional hacia las flores más grandes. En el estudio sobre el género funcional y su evolución temporal en *S. littorea*, encontramos que los individuos más femeninos no presentaron un mayor éxito reproductivo tanto en el cuajado de frutos y semillas como en el número total de semillas. Las medidas de variación del género fenotípico mostraron un continuo, con la mayoría de los valores cerca de 0,5; y ligeramente desviadas a la masculinidad cuando lo que se analizó fue el género funcional. Por último, encontramos una proporción de aproximadamente el 20% de plantas con pétalos blancos en dos poblaciones del norte de la Península Ibérica. El análisis del transcriptoma de los pétalos de distinto color reveló una expresión diferencial de algunos genes de la ruta biosintética de las antocianinas, cuya causa más probable parece ser la regulación de tipo *cis*. Asimismo, los datos bioquímicos apoyaron el bloqueo de la ruta.

ABSTRACT

Silene L. (Caryophyllaceae) shows a great variability of morphological and ecological characters. In particular, the high diversity of sexual systems and their evolution in addition to variations in flower color, are probably the most interesting features. In this thesis, we will review the main sexual strategies in *Silene* and estimate the frequency of the different sexual systems at the genus and subgenus level. We will then select section *Psammophilae* (Talavera) Greuter (endemic to the Iberian Peninsula and Balearic Islands) as our study case to analyze the sexual dimorphism in flowers, and the frequency, maintenance and reproductive advantages of the gynodioecious-gynomonoecious sexual system. Within this group, we will focus on *S. littorea* Brot. to study in detail variations in sex expression throughout the flowering period. Finally, we

will study the frequency and the biochemical and genetic bases of a petal color polymorphism (dark pink, light pink and white) in *S. littorea*.

The review of sexual systems in *Silene* showed that the most frequent sexual system is hermaphroditism (58%) followed by dioecy (14%), gynodioecy (13%) and gynodioecy-gynomonoecy (12%). These sexual systems are present in both subgenera, *Silene* and *Behenantha* (Otth) Endl., which indicates independent origins in both clades. Most populations of species of *Psammophilae* are gynodioecious-gynomonoecious; hermaphrodite plants are the most frequent, with a very low percentage of female plants in all populations. The study of sexual dimorphism and its consequences showed that hermaphroditic flowers were larger, produced more ovules and had higher pollen loads than female flowers. Moreover, selection gradients showed directional selection for larger flowers. During the study of sex expression and its temporal variations in *S. littorea*, we found that individuals with higher femaleness did not show a higher fruit set, seed set or total number of seeds. The phenotypic gender was continuous, with most values around 0.5, slightly skewed toward maleness when functional gender was taken into account. Lastly, we found a ~20% of white-flowered plants in two populations of the northern distribution of *S. littorea*. Transcriptome analysis of different color petals showed differential expression in some genes of the anthocyanin biosynthetic pathway; the most probable cause is a downregulation on *cis*-regulatory elements. In addition, the biochemical data support a blockage in the pathway.

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Chapter 1

Introduction

MOTIVATION

Species of the genus *Silene* L. (Caryophyllaceae) exhibit a great variety of morphological and ecological characteristics (Chater and Walters 1964, Talavera 1990, Morton 2005, Kephart 2006, Bernasconi et al. 2009). One of the most varied and bibliography-generating features of this genus is probably its diversity of mating and sexual systems, including among others, cleistogamy, prior autonomous selfing, hermaphroditism and dioecy (Desfeux et al. 1996, Jürgens et al. 2002, Davis and Delph 2005, Witt et al. 2013, Buide et al. 2015). Especially relevant is the study of how dioecy is originated in *Silene* (Darwin 1876, Baker 1963, Charlesworth 1999). Interest in the analysis of dioecy in *Silene* is possibly increased by the fact that some species show different sex determination types, including male or female heterogamety and neo-sex chromosomes (Slancarova et al. 2013, Weingartner and Delph 2014). The most frequent sexual systems in *Silene* are hermaphroditism, gynodioecy and dioecy (Jürgens et al. 2002). However, detailed studies have shown that gynomonoecious individuals (i.e. plants bearing hermaphroditic and female flowers) also exist in populations in a considerable number of species traditionally considered gynodioecious (e.g. Shykoff 1988, Talavera et al. 1996, Guitián and Medrano 2000, Lafuma and Maurice 2006, Dufay et al. 2010; Figure 1), demonstrating a gynodioecious-gynomonoecious sexual system. The present PhD thesis will first review the sexual strategies and frequency of sexual systems in *Silene*. The section *Psammophilae* was then chosen to explore some questions relating to the incidence, maintenance and ecological significance of gynodioecy-gynomonoecy. Next, we will study the sexual dimorphism and whether the flower size is under selection by pollinators in *Psammophilae* species. Finally, we will focus on sex expression of gynodioecious-gynomonoecious populations of *S. littorea*.

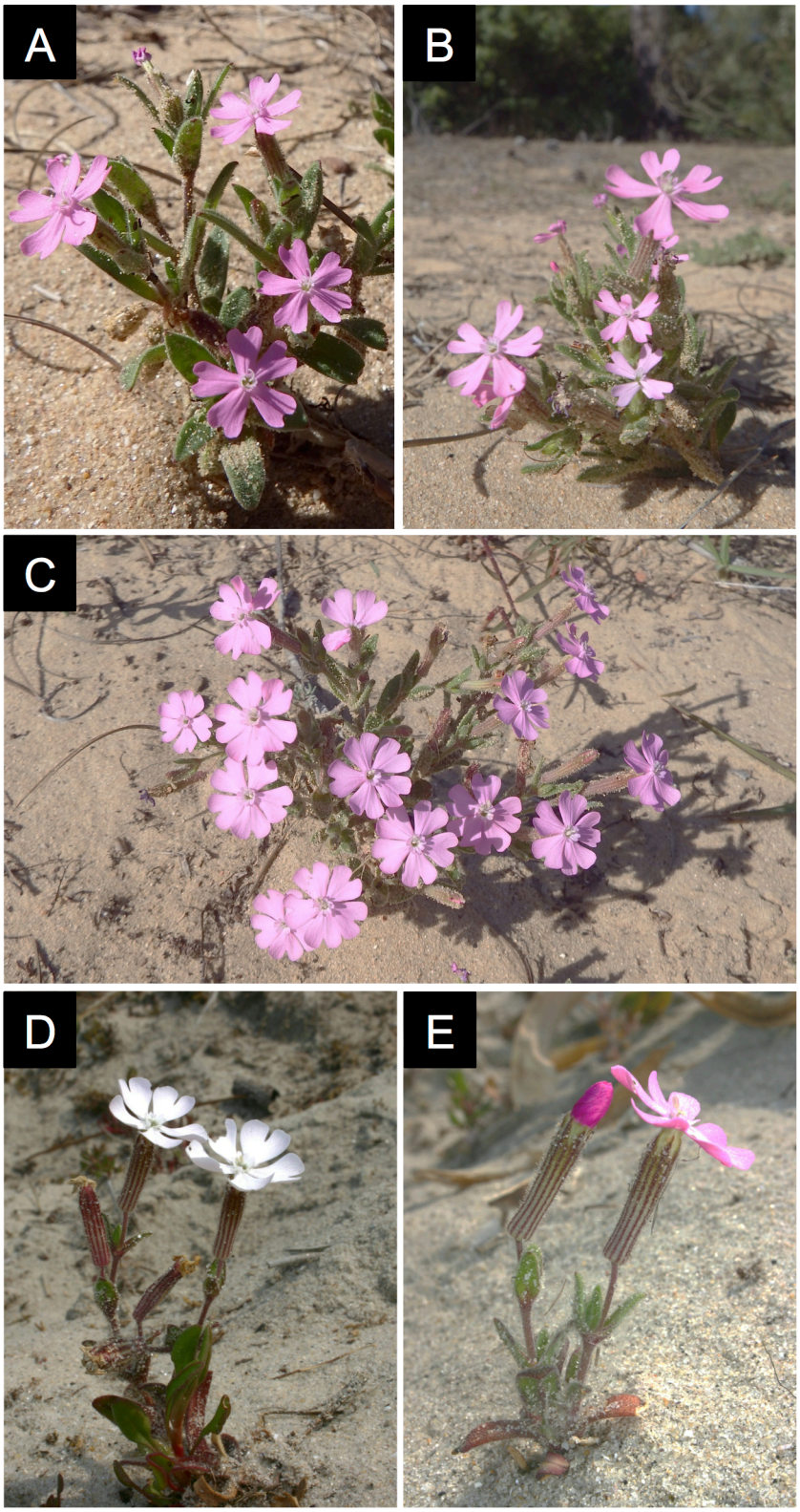

Figure 1. Female (A), gynomonoecious (B) and hermaphrodite (C) plants of *Silene littorea* from "Breña" population (Cádiz). (D) and (E) represent the petal color polymorphism of *Silene littorea* in "Barra" population (Pontevedra).

Another variable characteristic that has motivated a large number of studies is petal color. Undoubtedly, three main petal colors are most frequent in *Silene* species (in this order): pink, white, and red (Talavera 1990, Greuter 1995, Reynolds et al. 2009). These colorations and variations in intensities (e.g. light pink or magenta) are the result of a variable concentration and combination of three different anthocyanin derivatives: cyanidin, peonidin and pelargonidin (Kamsteeg et al. 1976, Kuwayama et al. 2005). Studies of petal color typically analyze the interspecific variability. For instance, Reynolds et al. (2009) showed how species with different petal colors are pollinated by totally different functional groups of pollinators. The studies of intraspecific variation in petal color within *Silene* species have received limited attention, and are only focused on how the hybrids between red and white petal color species segregate, and its implications for pollinators (Kamsteeg et al. 1979, Brothers and Atwell 2014, Page et al. 2014). Recently, Rahmé et al. (2014) studied the inheritance and reproductive consequences of the loss of anthocyanin in petals of polymorphic species *S. dioica*. However, the evolution of intraspecific variation (i.e. petal color polymorphism) and its implication in the speciation process has been highlight in recent years (Strauss and Whittall 2006, Rausher 2008, McKinnon and Pierotti 2010, Ortiz-Barrientos 2013). We have previously observed that *S. littorea* shows a flower color polymorphism in petals (Figure 1). Thus, we decided to analyze its frequency throughout its distribution area, and the biochemical and molecular basis of such polymorphism.

BACKGROUND

Evolution of sexual systems in flowering plants

The majority of angiosperm species are hermaphroditic, with the female and male function within the same flower. These sexual organs can be spatially (herkogamy) or temporally (dichogamy) separated in a flower to reduce the possibility of self-pollination, to benefit sexual specialization, or avoid physical interference (Bertin and Newman 1993). However, plants can also produce several flowers; consequently, female and male functions may be combined in multiple structural and temporal ways (Lloyd 1979, Barrett 2002). Thus, the separation of female and male functions in different individuals (dioecy) is an efficient mechanism to avoid self-fertilization and its most important consequence, inbreeding depression (Lloyd and Schoen 1992, Charlesworth 2006).

Different hypotheses have been proposed for the evolution of dioecy: via gynodioecy (Charlesworth and Charlesworth 1978), via androdioecy (Bawa 1980), through monoecy (Torices et al. 2011), via heterostyly (Muenchow and Grebus 1989, Pailler et al. 1998), and via heterodichogamy (Pannell and Verdú 2006). Figure 2 presents the most frequently referenced routes: gynodioecy, androdioecy and monoecy (these terms will be defined in Chapter 2). Theoretical models of the evolution of dioecy have been specially developed for the gynodioecy pathway, which is probably the best supported hypothesis for the evolution of dioecy (Charlesworth and Charlesworth 1978, Dufay et al. 2014). This pathway will be described and discussed in several chapters of this thesis. On the other hand, the evolution of dioecy via androdioecy requires the invasion of a female-sterile mutant as a first step. This step is very unlikely (Lloyd 1975, Charlesworth 1984, Pannell 2002, but see Billiard et al. 2015), however it may

occur in some genera: *Phillyrea* (Vassiliadis et al. 2000), *Fraxinus* (Wallander 2001), *Sagittaria* (Muenchow 1998). A more likely hypothesis for the evolution of androdioecy is the transition from dioecy, rather than a previous step toward dioecy (Figure 2; Pannell 2002, Wolf and Takebayashi 2004), where self-compatible hermaphrodites first invade a dioecious population followed by the disappearance of females. Models predict that self-fertilization is necessary for androdioecy to evolve from dioecy, and that it should evolve from hermaphroditism when androdioecious species are self-incompatible, as in the case of *Phillyrea angustifolia* and *Neobuxbaumia mezcalaensis* (Wolf and Takebayasi 2004).

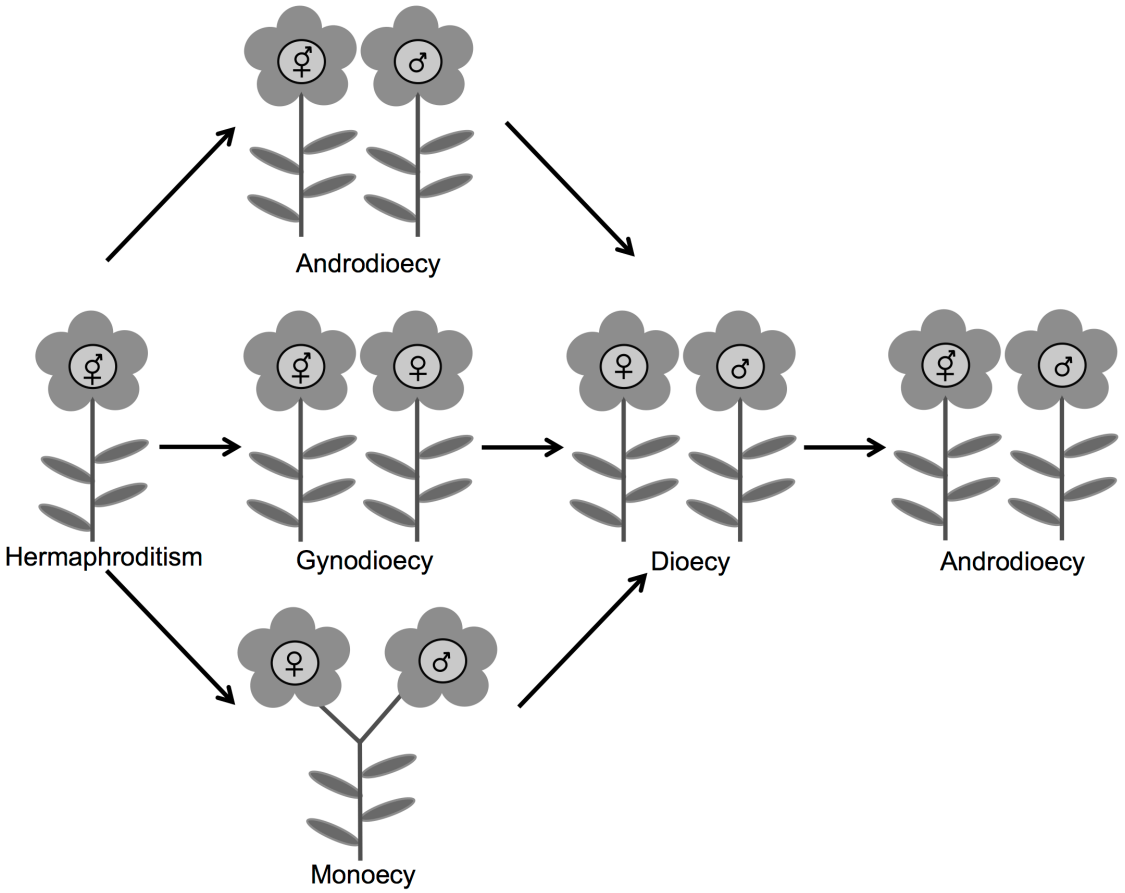

Figure 2. Three main pathways for the evolution of dioecy: via androdioecy (above), via gynodioecy (middle) and via monoecy (below). Androdioecy can also evolve from dioecy (see text). Flowers are depicted in dark grey and their gender is indicated in a light grey circle.

The monoecy pathway is better supported than the androdioecy pathway, although it is less common than evolution via gynodioecy (Figure 2; Dufay et al. 2014). It requires disruptive selection for female and male sex allocation in monoecious populations, which gradually increases gender specialization until unisexual plants originate (Barrett 2002). Evolution to monoecy can then occur through gynomonoecy or andromonoecy. However, theoretical models suggest the gynomonoecy pathway is unlikely, due to the high number of seeds that would require the female with respect to hermaphroditic flowers (de Jong et al. 2008). In contrast, Torices et al. (2011) found that the transition to monoecy through gynomonoecy was the most probable pathway in the Asteraceae family.

Flower color polymorphism and its genetic bases

Variations in flower color have attracted a great deal of attention since early days (e.g. Sprengel 1793, Mendel 1866). Flower color shifts between sister species have been extensively documented (e.g. *Antirrhinum*, *Aquilegia*, *Mimulus*, *Petunia*; Bradshaw et al. 1995, Hodges et al. 2002, Stuurman et al. 2004, Schwinn et al. 2006, see Narbona et al. 2014 for a review); but also within species (e.g. *Ipomoea purpurea*, *Iris lutescens*, *Mimulus lewisii*, *Mimulus aurantiacus*, *Parrya nudicaulis* or *Phlox drummondii*; Chang et al. 2005, Dick et al. 2011, Hopkins and Rausher 2011, Streisfeld et al. 2013, Wang et al. 2013, Wu et al. 2013). The co-occurrence of different color morphs in the same species and/or population is called polymorphism. The persistence of these polymorphisms over large spatial scales remains enigmatic. It is often attributed to genetic drift (Wright 1943). However, detailed field studies have revealed flower color polymorphisms maintained by balancing selection by pollinators or between pollinators and non-pollinator agents of selection, such as climate or herbivores (Epling and Dobzhansky 1942, Subramaniam and Rausher 2000, Warren and Mackenzie 2001,

Irwin and Strauss 2005, Eckhart et al. 2006, Strauss and Whittall 2006, Dick et al. 2011, Arista et al. 2013).

Anthocyanins, carotenoids and betalains (which replace anthocyanins in some groups of the order Caryophyllales, but not in Caryophyllaceae: Brockington et al. 2011) are the most common pigments responsible for petal coloration, and are widely distributed in angiosperms (Holton and Cornish 1995, Grotewold 2006). Other flavonoids apart from anthocyanins also give rise to flower colors such as the pale yellow aurones, but they are less common than anthocyanins (Davies 2009). Anthocyanins are the final product of the Anthocyanin Biosynthetic Pathway (ABP),

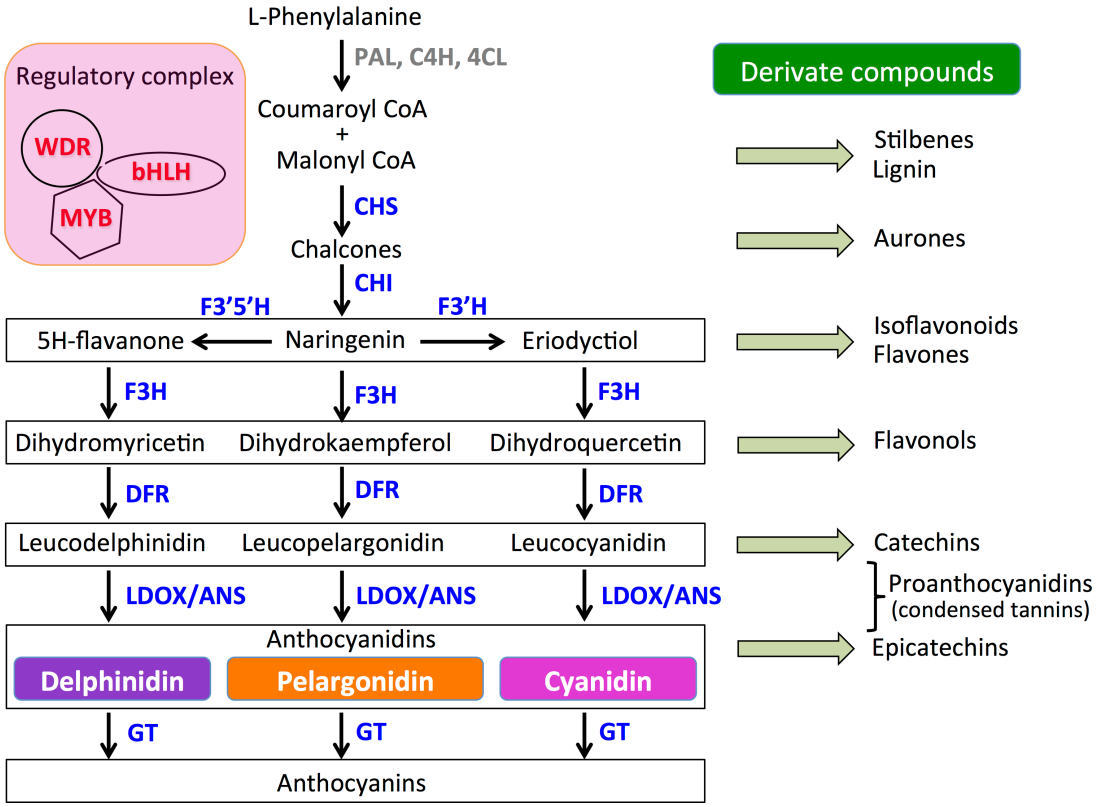

Figure 3. Scheme of the anthocyanin biosynthetic pathway (ABP). Core enzymes are depicted in blue, regulatory proteins in red and enzymes belonging to the phenylpropanoid pathway in grey. Green arrows indicate branch pathways leading to different secondary metabolites. Abbreviations: PAL: phenylalanil ammonia-lyase; C4H: cinnamic acid 4-hydroxylase; 4CL: coumarate CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; FNS: flavone synthase; F3'H: flavonoid 3'hydroxylase; F3H: flavanone-3-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol 4-reductase; LDOX/ANS: leucoanthocyanidin dioxygenase/anthocyanidin synthase; GT: glycosyl transferase. The three gene regulatory complex consists of a basic Helix Loop Helix protein (bHLH), WD Repeats (WDR) and R2R3-MYB domains (MYB) in most angiosperms. Image extracted and modified from Narbona et al. 2014.

with three main branches according to the final coloration: cyanidins produces pink-purple colors, delphinidins are responsible for blue coloration, and pelargonidins for red (Grotewold 2006). The production of one of these anthocyanins depends on the level of hydroxylation of the B-ring (Tanaka et al. 2008). Although anthocyanins are the primary pigmented product of the ABP, there are many pigmentless intermediates, including flavones, flavonols and proanthocyanidins. These intermediates provide protection against abiotic stresses such as extreme temperatures, drought, salinity and biotic agents like pathogens and herbivores (reviewed in Winkel-Shirley 2002, Davies 2009, Pollastri and Tattini 2011, Falcone Ferreyra et al. 2012). The enzyme-coding genes of the ABP and its associated regulatory genes are few and relatively well known, making this pathway a good object of study to analyze shifts in flower color at the genetic level (Figure 3; Grotewold 2006, Tanaka et al. 2008).

Evolutionary shifts from red to blue coloration or viceversa have been described in nature, but the most common change in flower color is the shift from pigmented to white flowers (Wheldale 1916, Rausher 2008). Wessinger and Rausher (2012) describe two types of mutation to explain the loss of anthocyanins: functional and regulatory. Functional mutations involve the blockage of at least one of the ABP coding enzymes; in contrast, regulatory mutations require: (a) a *cis*-regulatory mutation that downregulates any ABP enzyme, (b) loss of function of any of the regulatory enzymes (MYB, bHLH or WD40), or (c) a *cis*-regulatory mutation that suppresses one of these regulators. Spontaneous white flower mutants are common in nature, and researchers have found that both functional and regulatory mutations are responsible of the loss of anthocyanins (Sobel and Streisfeld 2013). Nevertheless, the evolutionary fixation of the white morph in populations seems to be related to regulatory mutations, specifically mutations affecting the MYB transcription factor, due to the capacity for specific-tissue

regulation, avoiding the pleiotropic effects of anthocyanin loss in vegetative tissues (see Wessinger and Rausher 2012).

Phylogeny and taxonomy of Silene *and section* Psammophilae

Caryophyllaceae is a large family composed of some 86 genera and 2200 species distributed across the globe (Smissen et al. 2002). The monophyly of the Caryophyllaceae is supported by molecular and morphological data (Cuénoud et al. 2002, Judd et al. 2002, Greenberg and Donoghue 2011). It has been traditionally subdivided in three subfamilies (Bittrich 1993): Alsinoideae, Caryophylloideae and Paronychioideae. However recent molecular studies have demonstrated that these subdivisions are paraphyletic (Cuénoud et al. 2002, Smissen et al. 2002, Harbaugh et al. 2010), and suggest the subdivision of Caryophyllaceae in 11 tribes instead of subfamilies (Figure 4; Fior et al. 2006, Harbaugh et al. 2010, Greenberg and Donoghue 2011). This way, Caryophylloideae is now composed by tribes Caryophylleae (e.g. *Dianthus*, *Petrorrhagia*, *Velezia*) and Sileneae (e.g. *Silene*, *Agrostemma*, *Lychnis*, *Petrocoptis*) (Figure 4; clades D and E; Halbourgh et al. 2010).

Within tribe Sileneae, phylogenetic relationships have been also controversial (e.g. see Greuter 1995). More recently, Oxelman et al. (2001) considered the subdivision of Sileneae in eight genera (*Agrostemma* L., *Atocion* Adans., *Eudianthe* (Rchb.) Rchb., *Heliosperma* Rchb., *Lychnis* L, *Petrocoptis* A. Braun ex Endl., *Silene* L. and *Viscaria* Bernh.). Recent molecular studies supported this classification (Popp and Oxelman 2004, 2007, Erixon and Oxelman 2008). From all of these genera, *Silene* is the most diverse, with ca. 700 species (Mabberley 2008), and is subdivided into two phylogenetically supported subgenera: *Silene* and *Behenantha* (Othh.) Endl. (Figure 5; Rautenberg et al. 2010).

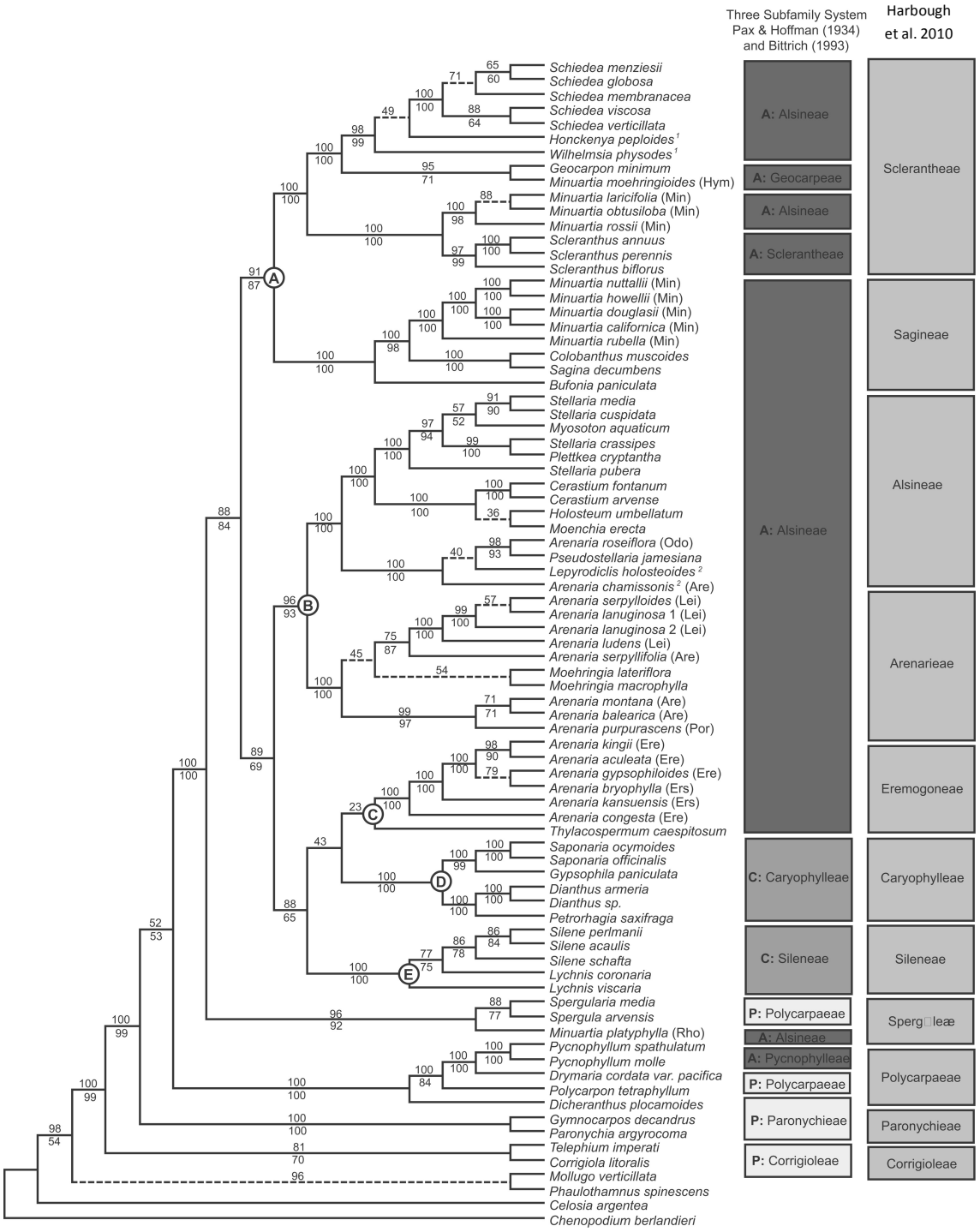

Figure 4. Maximum likelihood (ML) phylogeny of Caryophyllaceae extracted from analysis of combined *mat*K, *trn*L-F and *rps16* sequences. Numbers above the branches represent ML bootstrap, while those below are for maximum parsimony. Extracted from Harbaugh et al. 2010.

Section *Psammophilae* (Talavera) Greuter belongs to subg. *Behenantha* (Figure 5; Erixon and Oxelman 2008, Rautenberg et al. 2010, Oxelman et al. 2013), and it was previously considered a subsection within section *Erectorefractae* Chowdhuri together with subsection *Erectorefractae* (*S. almolae* and *S. germana*) (Talavera et al. 1979).

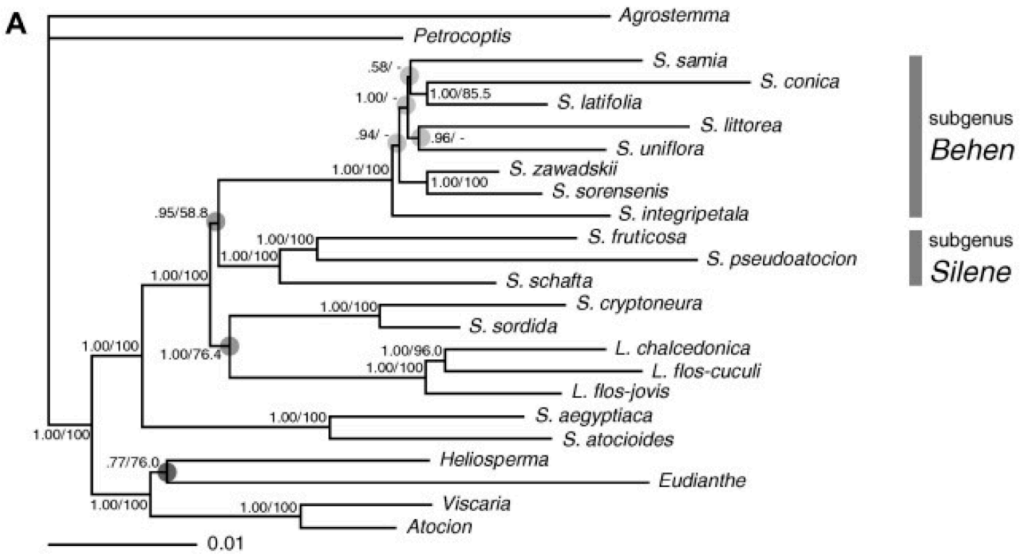

Figure 5. Mayority rule consensus tree from Bayesian analysis of *Silene* species showing *Silene littorea* belonging to subgenus *Behen* (Moench)(= *Behenantha* (Otth.) Endl.). Numbers on nodes indicate branch support (Bayesian posterior probability/Parsimony bootstrap). Extracted from Erixon and Oxelman 2008.

However, Greuter (1995) considered the section status due to differences in cell shape and flowering rhythm, in addition to the differences previously described by Talavera et al. (1979) (e.g. monochasium inflorescences in *Psammophilae* and dichasium in *Erectorefractae*). *Silene pendula* L. was previously included in Section *Psammophilae*, but it was placed in *Silene* sect. *Behenantha* Otth by Oxelman et al. (2013), related to other species such as *S. vulgaris* (Erixon and Oxelman 2008, Rautenberg et al. 2010). In addition, our preliminary analysis with molecular data based on ITS suggested that *S. psammitis* Link, *S. littorea* Brot., *S. stockenii* Chater, *S. cambesedessii* Boiss and Reut. and *S. adscendens* Lag. form a well supported clade, although *S. germana* is outside this group, in an unresolved polytomy (Figure 6). Thus, section *Psammophilae* is composed by five species (*Silene adscendens*, *S. cambessedesii*, *S. littorea*, *S. psammitis* and *S. stockenii*). The species status was considered for *S. adscendens* (e.g. Chater and Walters 1964), sometimes considered a subspecies of *S. littorea*, e.g. Talavera 1990).

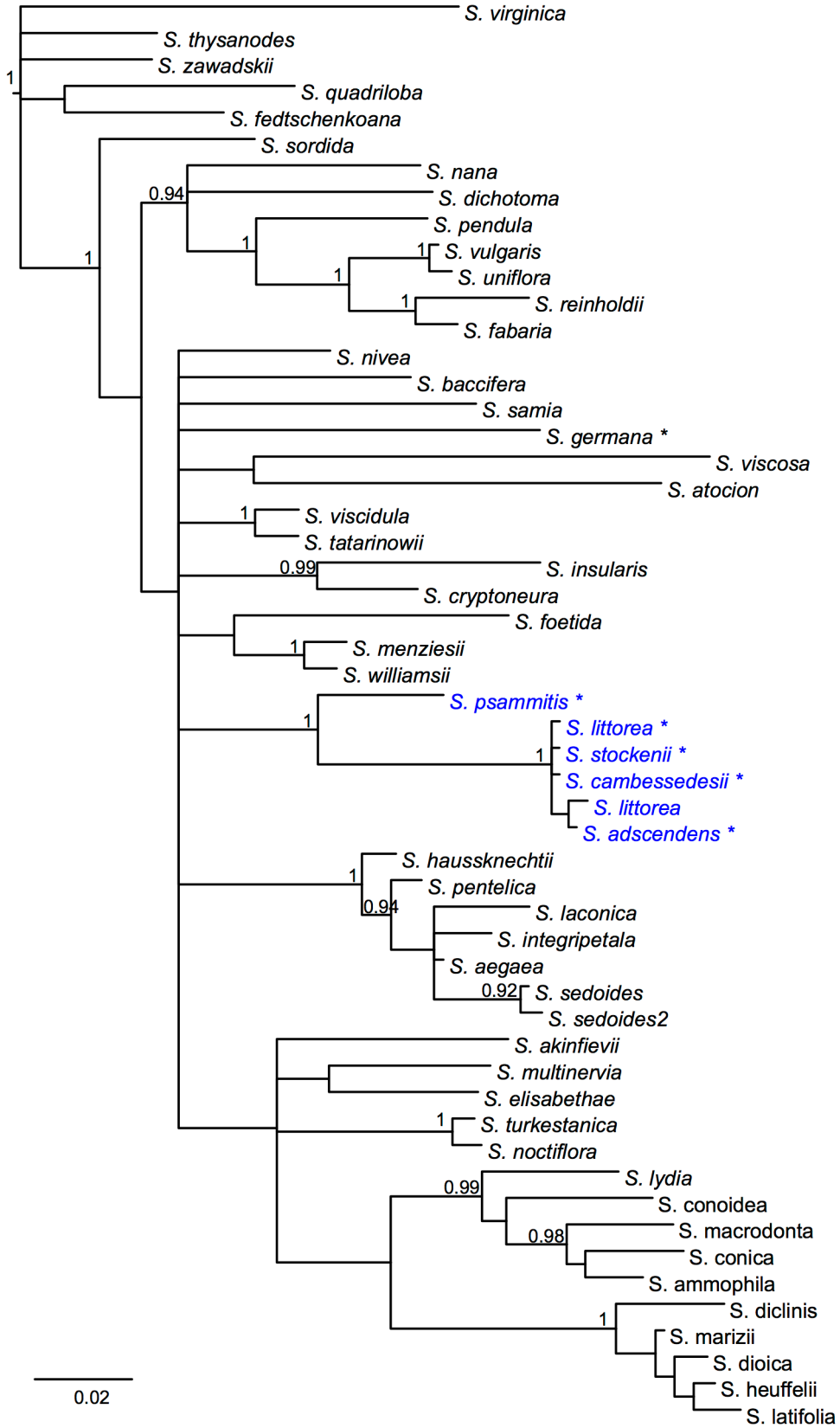

Figure 6. Majority rule consensus tree of ITS of *Silene* species resulting from two independents runs in MrBayes for 5.000.000 generations after removing the 20% of the trees as burn-in. Posterior Probabilities greater than 0.9 are indicated above branches. We applied the GTR+G model of sequence evolution. Asterisks denote sequences newly obtained for this study. The rest were downloaded from NCBI GenBank.

Study species

Species of section *Psammophilae* are annuals, self-compatible and endemic to the Iberian Peninsula and Balearic Islands. They are pubescent with patent glandular and appressed eglandular hairs (Talavera 1990). The inflorescence is a monochasium or solitary flowers (Figure 7). Flowers are pink, rarely white; in *S. stockenii* they are red on the underside of the petals (Figure 7). They are open during the day and night, when sometimes emit floral scents. Pedicels are erect and tend to be curved in fruit, which is a capsule. Seeds are reniform, with flattened or slightly concave (*S. cambessedesii*, *S. psammitis*) or convexe and coliculate (*S. littorea*, *S. adscendens*, *S. stockenii*) side surfaces (Candáu and Talavera 1979, Ocaña et al. 2011). Pollination is mainly entomophilous, although low levels of autonomous self-pollination may exist (Talavera et al. 1996, Hidalgo-Triana 2010). The main pollinators of *S. littorea* are species of Bombylidae, Sphingidae, Syrphidae and Lycaenidae (personal observations). In *S. stockenii* only species of Bombylidae have been recorded (Talavera et al. 1996).

Silene littorea and *S. cambessedesii* grow at the sea level on coastal sand dunes ecosystems from the Iberian Peninsula and Balearic Islands, respectively (Figure 8). *Silene adscendens* is endemic to the Almeria province and grows on banks of temporary streams, from zero to 300 m above sea level. *Silene psammitis* grows on dolomites or slates between 300 m and 1500 m above sea level, and is amply distributed in the Iberian Peninsula although its populations are dispersed and most times with low number of individuals. Lastly, the endangered *S. stockenii* grows on calcareous soils at ca. 200 m of altitude from the southern peninsula (Cádiz province). Only four small populations are known (Talavera et al. 1996), and its habitat is seriously threatened by quarries and crops (Bañares et al. 2004). Most aspects of the sexual system and the

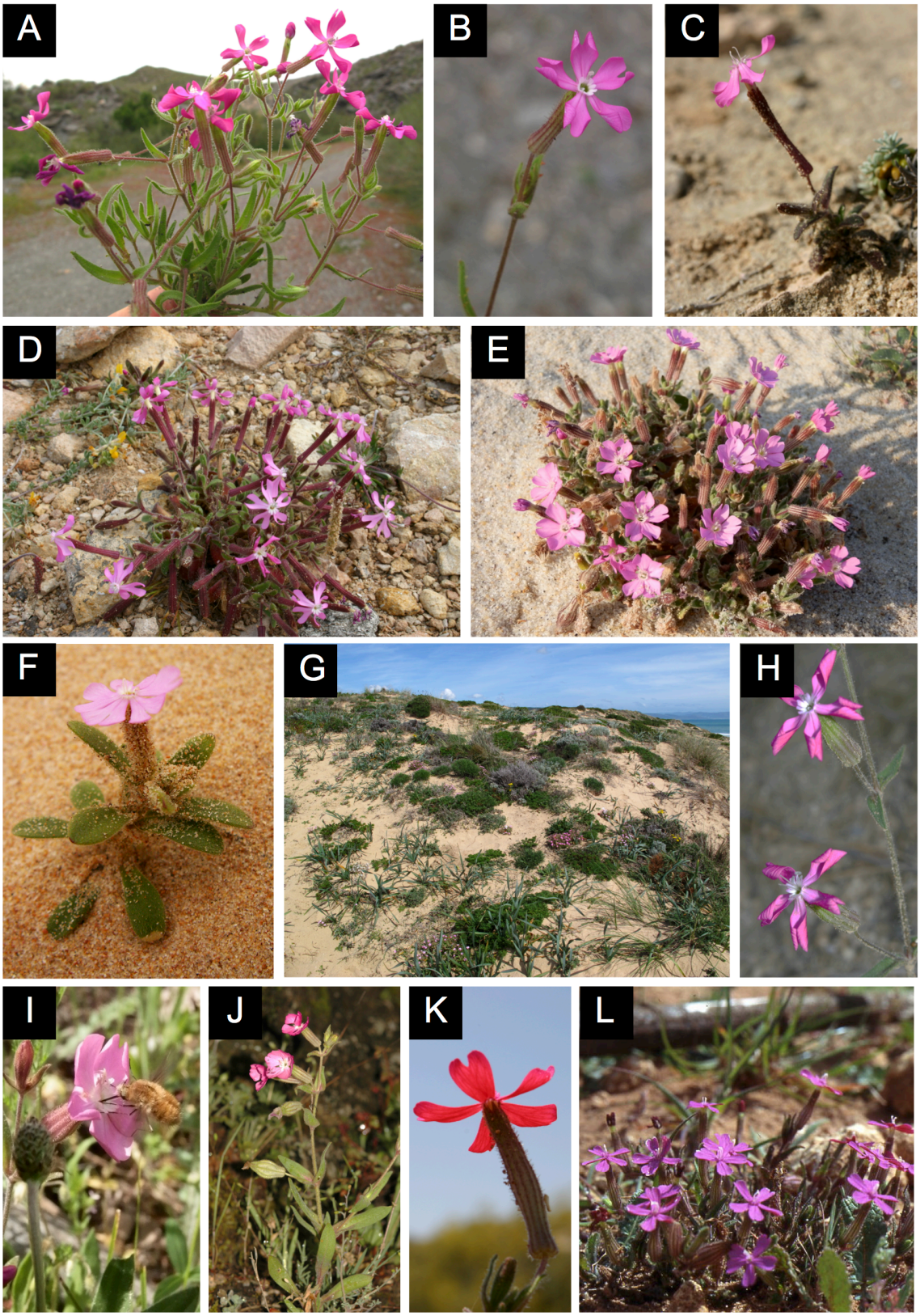

Figure 7. Plants and flowers of species of section *Psammophilae*. (A, B) *Silene adscendens*, (C, D) *S. cambessedesii*, (E, F) *S. littorea*, (G) Habitat of *S. littorea* in "Trafalgar" population, (H-J) *S. psammitis*, (K, L) *S. stockenii*.

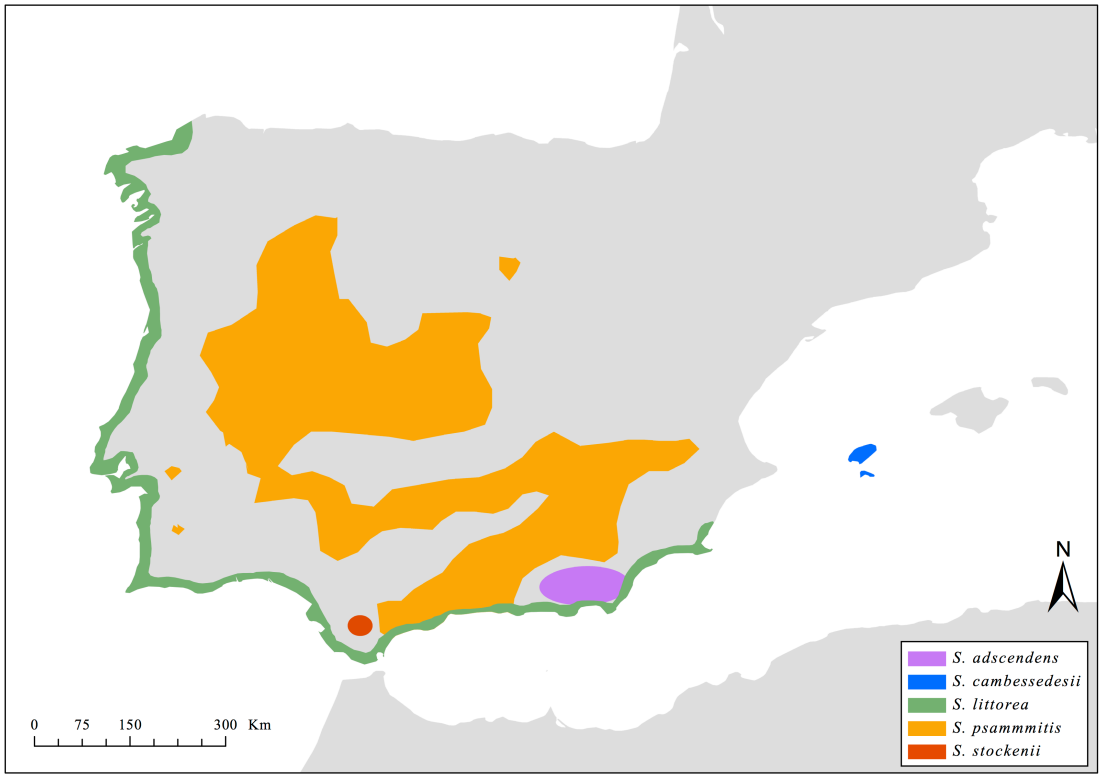

Figure 8. Distribution area of species of section *Psammophilae*: *S. adscendens* (purple), *S. cambessedesii* (blue), *S. littorea* (green), *S. psammitis* (orange) and *S. stockenii* (red).

reproductive biology of *S. stockenii* have been previously analyzed in Talavera et al. (1996); thus, this species will not be studied in the current thesis.

GENERAL AND SPECIFIC AIMS

The general objective of this dissertation is to explore the diversity of sexual strategies in *Silene*, and the study of sexual system and flower color polymorphism in species of section *Psammophilae*. We will specifically try to answer the following questions:

1. What are the main aspects related to sexual strategies, including evolutionary pathways of sexual system and sex determination, sex and gender expression, and sexual dimorphisms in *Silene*?
2. How frequent are the sexual systems in species of *Silene* at the genus and subgenus level?
3. Which is the principal sexual system of the species of *Silene* section *Psammophilae*? Do they vary among populations?
4. What is the frequency of different morphs and types of flowers (hermaphrodite or female) within populations?
5. How do hermaphroditic and female flowers of species of section *Psammophilae* differ in morphology, ovule and pollen production?
6. Is flower size under pollinator selection?
7. Do female individuals have some advantage over hermaphrodites to maintain them in populations?
8. How does sexual expression of *S. littorea* vary throughout the flowering period?
9. How frequent is petal color polymorphism in *S. littorea*?
10. Which biochemical compounds are responsible for flower color in *S. littorea*?
11. Which are the most probable genetic mechanisms causing flower color polymorphism?

THESIS STRUCTURE

The thesis manuscript has been divided into seven chapters. The **current chapter** provides a general introduction that includes the motivation, background (evolution of sexual systems in angiosperms, flower color polymorphisms, Caryophyllaceae phylogeny), main objectives, structure and a list of papers generated by this thesis. Chapters 2, 3, 4 and 5 are manuscripts, which are published or in the process of publication. **Chapter 2** focuses on the genus *Silene*, describing the most important findings on sexual strategies in *Silene*, including some of the most important results from Chapters 3 and 5 (Objective 1). In **Chapter 3**, the frequency of different sexual systems at the genus and subgenus level is estimated based on a survey of the literature. The sexual system of *Psammophilae* species is described in several populations, and estimates of the frequency of sexual morphs and types of flowers within populations are given (Objectives 2, 3, 4 and 7). **Chapter 4** focuses on flower dimorphism shown between hermaphroditic and female flowers, and what are the most important consequences for the maintenance of the sexual system in *Psammophilae* species (Objectives 5, 6 and 7). In **Chapter 5**, sexual expression in two populations of *Silene littorea* is studied throughout the flowering period. Here we use a continuous measure for sex expression, and analyze if those individuals with higher femaleness have greater fitness than those with less femaleness (Objectives 7 and 8). In **Chapter 6**, we study the presence of a flower color polymorphism in *S. littorea* along the Iberian coast, and identify the most probable genetic causes of this flower color polymorphism. To this end, we first used a transcriptome analysis to sequence and measure the expression of several genes in the anthocyanin biosynthetic pathway; we also examined the petal biochemistry with HPLC (Objectives 9, 10 and 11). Finally, **Chapter 7** is a general discussion of the main findings of this thesis and its main conclusions.

LIST OF PAPERS GENERATED IN THIS THESIS

1. Casimiro-Soriguer I, Buide ML, Narbona E (2013) The roles of female and hermaphroditic flowers in the gynodioecious-gynomonoecious *Silene littorea*: insights into the phenology and sex expression. *Plant Biology* 15:941-947.
2. Casimiro-Soriguer I, Buide ML, Narbona E (2015) Diversity of sexual systems within different lineages of the genus *Silene*. *AoB Plants* doi: 10.1093 /aobpla/plv037.
3. Casimiro-Soriguer I, Narbona E, Buide ML (2016) Diversity and evolution of sexual strategies in *Silene*: a review. *Progress in Botany*, Vol. 77. Berlin: Springer. *In press*.
4. Casimiro-Soriguer I, Narbona E, Buide ML, del Valle JC, Whittall JB. Transcriptome and biochemical analysis of a flower color polymorphism in *Silene littorea* (Caryophyllaceae) (submitted to *BMC Genomics*).
5. Casimiro-Soriguer I, Narbona E, Buide ML, Arista M. Flower dimorphism and selection on flower size in four gynodioecious-gynomonoecious *Silene* species (manuscript).

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2

Chapter 2

Diversity and evolution of sexual strategies in *Silene*: a review

Casimiro-Soriguer I, Narbona E, Buide ML (2015) Diversity and evolution of sexual strategies in *Silene*: a review. *Progress in Botany*, Vol. 77, *in press*.

ABSTRACT

The variety and evolution of reproductive strategies in plants has attracted the attention of scientists for a long time. The genus *Silene* has been the focus of several studies related to the diversity and evolution of sexual systems. This review will summarize the huge amount of knowledge on sexual strategies in *Silene* species. Hermaphroditism is the most frequent condition in *Silene*; however, there is a relatively high frequency of gynodioecy and dioecy compared to angiosperms and dicotiledons. In some gynodioecious species, gynomonoecious individuals are common, forming a gynodioecious-gynomonoecious sexual system that is rare among angiosperms. Dioecy has independently evolved in the two phylogenetically supported subgenera of *Silene*, with a probable origin down the "gynodioecious pathway". Heterogametic sex chromosomes have made *S. latifolia* and other dioecious species of the genus important models for the evolution of sex determination. In *Silene* species, studies on sexual expression at the plant and population level suggest that it is highly variable. Sexual dimorphism in reproductive and vegetative characters of dioecious species showed patterns that generally fit those found in other species. Compared with other genera of angiosperms, *Silene* presents a unique opportunity to evaluate: the evolution of the different sexual systems and sex chromosomes (being of the few angiosperm genera with female heterogamety), the maintenance of gynodioecious and gynodioecious-gynomonoecious sexual systems, and the evolutionary implications of sexual dimorphism.

INTRODUCTION

The great variety of breeding systems in plants has been deeply interesting to scientists since the nineteenth century (Darwin 1877, Müller 1883). In fact, studies of sexual systems in contemporary scientific literature are on the increase, particularly from 1995 on (Fig. 1). How this genetic and morphological diversity has emerged from the ancestral condition of hermaphroditism is an important question, which has been widely discussed (Charlesworth and Charlesworth 1978, Barrett 2013, Crossman and Charlesworth 2014). Many intermediate steps between hermaphroditism and dioecy can be found in nature (e.g. gynodioecy or monoecy) and their study has attracted the attention of researchers over the years (Spigler and Ashman 2012, Golenberg and West 2013, Dufay et al. 2014). One of the main questions is why the separation of sex may evolve and how it may be maintained. This question has been tested in gynodioecious species: females have the advantage of avoiding inbreeding, whereas hermaphrodites may represent a bet-hedging strategy (Pettersson 1992).

Silene is the largest and most diverse genus of Caryophyllaceae (Mabberley 2008), with about 700 species (Mabberley 2008, Oxelman et al. 2013). It is a model system for studies in ecology and evolution, especially the ecology of biotic interactions and the evolution of sex chromosomes in plants (Bernasconi et al. 2009). Species of *Silene* show a high diversity of sexual systems (Desfeux et al. 1996, Jürgens et al. 2002, Casimiro-Soriguer et al. 2015), including dioecious species with sex chromosomes (e.g. *S. latifolia*). All species are self-compatible in *Silene*, and some of them are even cleistogamous (Jürgens et al. 2002, Witt et al. 2013, and references therein). Individuals or flowers may show spatial (e.g. dioecy, gynodioecy or gynomonoecy) and temporal separation (e.g. protandry in hermaphroditic flowers) of the male and female functions, which could help avoid the effects of inbreeding depression (Charlesworth 1999, but see Davis and Delph 2005 and Reynolds et al. 2009) or

allocating different resources to female and male functions (Lloyd 1979, Pettersson 1992, Delph 2003).

Here we will review the variety of sexual systems in *Silene* and the possible evolutionary pathways that could lead to this diversity. We summarize the advances in sex determination in dioecious and non-dioecious species. The variations in sex and gender expression will then be analyzed at the flower, individual, and population levels. Finally, sexual dimorphism in secondary sexual characters will be addressed for both reproductive and vegetative traits in species with two types of flowers. This review attempts to collate the huge amount of data on the evolution of gender and sexual systems in *Silene*. This may provide a valuable opportunity to generate and test hypotheses regarding the evolution and maintenance of certain sexual strategies that have repeatedly evolved in plants.

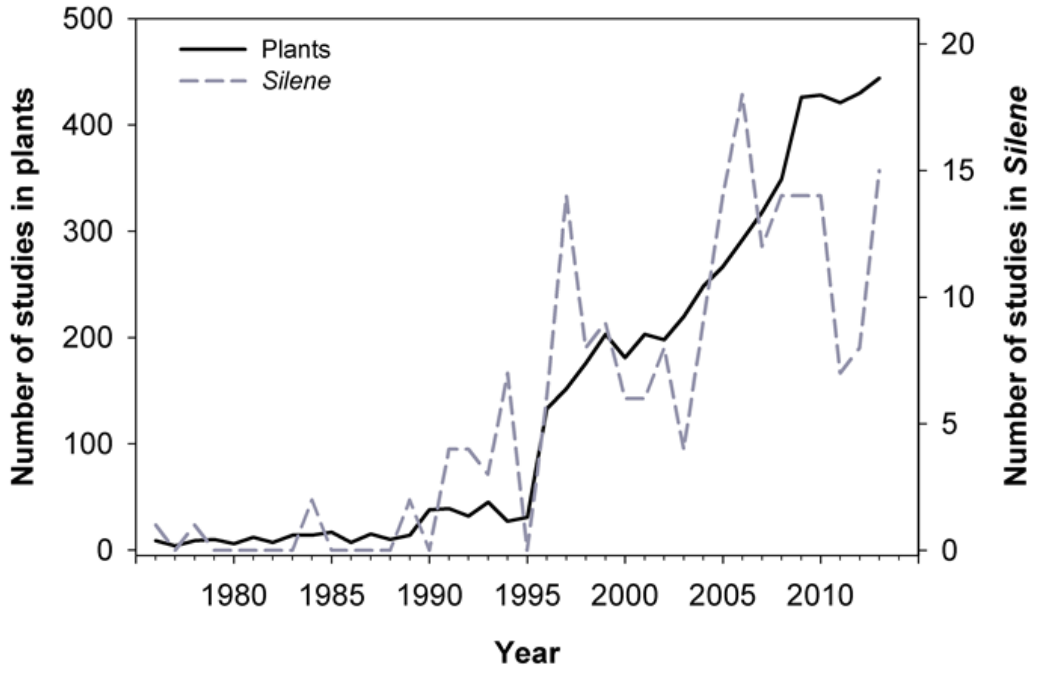

Figure 1. Studies of sexual systems in plants (black line) and in the genus *Silene* (grey dashed lines). We performed a literature search in SCOPUS from 1976 to 2013 for scientific papers including at least one of the following words: sexual system, breeding system, hermaphroditic, hermaphrodite, hermaphroditism, dioecious, dioecy, gynodioecious, gynodioecy, gynomonoecious, gynomonoecy, androdioecious, androdioecy, andromonoecious, andromonoecy, monoecious, monoecy. We limited the results to the area of life sciences restricted to the subject areas of agriculture and biological sciences, biochemistry, genetics and molecular biology, environmental sciences, multidisciplinary and earth and planetary sciences. We found a total of 5645 papers on plants as a whole, and 210 when restricting the search to genus *Silene*.

DIVERSITY AND DEFINITION OF SEXUAL SYSTEMS

Over time, the studies on plant reproductive strategies and sexual systems have generated a variety of terms to describe reproductive systems, but they are often inconsistently used. To avoid confusion, we therefore start this review by defining the most important terms relating to sexual systems.

Breeding, mating and sexual systems have been inconsistently defined in the literature (Neal and Anderson 2005). Neal and Anderson (2005) suggest the use of the term "breeding system" for the distribution of sex within and among plants (e.g. gynodioecy), for physiological aspects (e.g. self-incompatibility) and for morphological differences within populations (e.g. heterostyly); and "mating system" when the genetic relatedness and pairings between individuals are examined (e.g. selfing rate). We accept their definition of breeding system, but consider the term "sexual system" more appropriate for the distribution of sex within and among plants. Therefore, "breeding system" should not be considered a synonym for sexual system, as some authors have defined (e.g. Lloyd 1979, Harder and Barrett 2006).

The distribution of sexual organs can be considered on different levels: (1) flower, (2) plant and (3) population. (1) At the flower level, female and male sexual organs can be located in the same (hermaphroditic or perfect flower) or different flowers (female or pistillate and male or staminate flowers). (2) At the plant level, an individual is considered hermaphrodite when it bears only hermaphroditic flowers. When there are different types of flowers on the same individual, the suffix –monoecious or –monoecy is used. Monoecy itself occurs when the same plant produces both pistillate and staminate flowers. The term andro(gyno)monoecy or trimonoecy is applied to plants bearing perfect plus staminate(pistillate) flowers or all three types, respectively. When different sexes of flowers are distributed among different individuals, the suffix –dioecious or –dioecy is used. (3) On the population level, we can define populations as monomorphic, dimorphic or polymorphic.

Monomorphic populations have only one type of plants (i.e. hermaphroditic, monoecious, andromonoecious or gynomonoecious). In dimorphic populations, there are two types of plants: e.g. males and females (dioecy), females and hermaphrodites (gynodioecy) or males and hermaphrodites (androdioecy). In polymorphic populations there are more than two types of plants, e.g. males, females and hermaphrodites (trioecy, Fleming et al. 1994, Cruden and Lloyd 1995). Please note that unfortunately, Harder and Barrett (2006) also applied the term subdioecy to trioecious species. We advise against this, and prefer to follow Bailey and Delph (2007a), who consider subdioecy close to dioecy, with a high frequency of females and hermaphrodites achieving nearly all their fitness via pollen, but retaining the possibility of reproducing through seeds. On the other hand, some sexual systems that have been considered dimorphic at the population level show other intermediate morphs. Thus, some gynodioecious populations include hermaphroditic individuals bearing also female flowers (i.e. gynomonoecious individuals). The correct term for this sexual system is gynodioecy-gynomonoecy, in which females, hermaphrodites and gynomonoecious plants coexist within a population (Desfeux et al. 1996).

SEXUAL SYSTEMS IN *SILENE*

As in the case of studies of sexual systems in plants as a whole, studies of sexual systems in *Silene* have shown an abrupt increase in the last twenty years, with nearly 60% thereof published in the last decade (Fig. 1). Interestingly, from the 210 studies of sexual systems in *Silene* that we found in our search (see details in Fig. 1), 87% were focused on three species only: the dioecious *S. latifolia* (63%), the gynodioecious *S. vulgaris* (14%), and

Table 1. Percentage of sexual systems in species *Silene*, angiosperms and dicotyledons in general, and Hawaiian and Levant Floras.

Sexual system	*Silene*[a]	Angiosperms[b]	Dicotyledons[c]	Hawaiian Flora[d]	Levant Flora[e]
Hermaphroditism	58.2	72	71	62.4	86.6
Dioecy	14.3	4	4	14.7	2.2
Gynodioecy	25.5	7	*	3.8	0.3
Monoecy	-	5	4	7.6	3.6
Gynomonoecy	1.0	3	7	3.9	0.4
Andromonoecy	1.0	*	**	4.5	5.7
Others	-	9	14	-	1.1

[a] Percentage of *Silene* species calculated from data of 98 species revised by Casimiro-Soriguer et al. 2015. In the case of species with variable sexual system within or among populations, the most frequent sexual system was used (for a similar sexual system classification, see Jürgens et al. 2002). The available percentages data of other groups were taken from Richards 1997[(b)], Yampolsky and Yampolsky 1922[(c)], Sakai et al. 1995[(d)], de Jong et al. 2008[(e)]. * sexual system included in the group "others". ** sexual system included in the group "gynomonoecy".

the sexually variable *S. acaulis* (10%), in which gynodioecious, trioecious, subdioecious and dioecious populations have been described (Maurice et al. 1999, Alatalo and Molau 2001). The remaining 13% of the studies included eight other non-dioecious species (9%) and another three dioecious species (*S. dioica*, *S. otites* or *S. diclinis*; 4%). Overall, studies in *Silene* are skewed toward dioecious species, and especially in *S. latifolia*; however, dioecy is the third most common sexual system in the genus after hermaphroditism and gynodioecy (see below). This result is not surprising considering the importance of *S. latifolia* in the study of the evolution of sex chromosomes (see Sect. 5) and sexual dimorphism (see Sect. 7), among other aspects (Bernasconi et al. 2009).

Hermaphroditic, gynodioecious, gynomonoecious, dioecious, trioecious, and even andromonoecious species have been reported in the genus *Silene* (revised in Jürgens et al.

2002 and Casimiro-Soriguer et al. 2015). Despite *Silene* is a large genus (~700 species), there is a relatively important number of species in which the sexual system have been specifically studied or described (98 species, Casimiro-Soriguer et al. 2015). Based on these data, the most frequent sexual system in *Silene* is hermaphroditism (58.2%, Table 1). However, the proportion of hermaphrodites is clearly lower than the estimate for all angiosperms, and dicotyledons. In comparison with floras with available frequencies of the different sexual systems, the frequency of hermaphroditism in *Silene* is lower than those of the species of Levant flora (Israel) and similar to the Hawaiian flora. Gynodioecy (25.5%) is the second most frequent sexual system, more than three times higher than for angiosperms and dicotyledons as a whole, and higher than in the Levant local floras. In the genus *Silene*, gynodioecy-gynomonoecy occurs frequently (Talavera et al. 1996, Desfeux et al. 1996, Jürgens et al. 2002, Dufay et al. 2010, Casimiro-Soriguer et al. 2013), although is sometimes classified as gynodioecy (e.g. Lafuma and Maurice 2006). In Table 1, we categorized all those species with gynodioecious-gynomonoecious sexual systems as gynodioecious for simplicity (~50% of the cases, Casimiro-Soriguer et al. 2015). The frequency of dioecy also was more than three times higher than angiosperms and dicotyledons as whole, although very similar to the Hawaiian flora. To our knowledge, monoecious species do not occur in *Silene* (Casimiro-Soriguer et al. 2015); however, monoecy has a relative importance in the different datasets (between 3.6 and 7.6%). Although gynomonoecy and andromonoecy are present in the genus, the frequency of these sexual systems is very low (Table 1). The only gynomonoecious species was *S. noctiflora* (Folke and Delph 1997, Davis and Delph 2005), although populations with hermaphrodites and gynomonoecious plants have also been described (Jürgens et al. 2002). Gynomonoecy in *Silene* is to be expected, due to its combination with other sexual systems such as gynodioecy (i.e. gynodioecious-gynomonoecious species) given the possible related genetic determination system (Garraud et al. 2011). Andromonoecy has

only been reported in *S. tibetica* (Oxelman et al. 2001), but there are no more studies that confirm the sexual system of this species.

Silene was early known for its variety of sexual systems (Müller 1883, Knuth 1908); nevertheless, different sexual systems such as hermaphroditism or dioecy (as gynodioecy or gynomonoecy) are rarely described in checklists, national or local floras, or scientific papers. In fact, Desfeux et al. (1996) already noted that gynodioecy was usually not considered in floras. Thus it is reasonable to think that a non-negligible number of *Silene* species, usually described as hermaphrodites in floras, have other sexual systems. Accordingly, gynodioecy or gynomonoecy may be underestimated in the percentages shown in Table 1, because of the relatively low percentage of species with well-known sexual systems (~14%, Casimiro-Soriguer et al. 2015). Conversely, most dioecious species are probably already described due to their easy identification; therefore, the percentage would decrease when more species were included in the dataset.

EVOLUTIONARY PATHWAYS OF SEXUAL SYSTEM AND SEX DETERMINATION IN *SILENE*

Although hermaphroditism is the commonest sexual system in angiosperms, dioecy has evolved many times in angiosperm phylogeny (Weiblen et al. 2000, Dufay et al. 2014). The two main evolutionary pathways proposed to explain the origin of dioecy are gynodioecy and monoecy (Charlesworth and Charlesworth 1978, Barrett 2002, Golenberg and West 2013). Dioecy may also be a transition to the rare androdioecy, as has been hypothesized for *Mercurialis annua* (Pannell 1997, Pannell et al. 2004). There is another less-considered pathway that renders dioecy from distily (Muenchow and Grebus 1989). Although dioecy has been suggested to have evolved through monoecy in different groups (Renner and Won 2001, Torices et al. 2011), the gynodioecy pathway is generally more supported given the

appreciable number of gynodioecious species that are related to dioecious species (Charlesworth and Charlesworth 1978, Maurice et al. 1993, Dufay et al. 2014).

Although *Silene* is a large genus without a complete resolved phylogeny, it has been divided into the well-supported phylogenetic subgenus *Behenantha* and subgenus *Silene* (Popp and Oxelman 2004, Rautenberg et al. 2010). Dioecious species have been placed in these two clades: *S. latifolia* and dioecious relatives in subgenus *Behenantha*, and *S. otites* and relatives in subgenus *Silene* (Desfeux et al. 1996, Marais et al. 2011, Slancarova et al. 2013). *Silene* also has many gynodioecious species (Table 1), which is uncommon in other genera of the Caryophyllaceae (Matsunaga et al. 2003). Desfeux et al. (1996) mapped the evolution of sexual systems in a phylogeny of 22 species of *Silene* and suggested that gynodioecy was the ancestral condition of this genus. Fifteen years later, using high-resolution molecular tools, Marais et al. (2011) found that the most probable ancestral condition is either gynodioecy or hermaphroditism. Independently of the ancestral sexual system, it seems more likely that dioecy evolved via gynodioecy (Marais et al. 2011). Recently, it has been found that dioecy, hermaphroditism, gynodioecy and gynodioecy-gynomonoecy are present with a similar frequency in both subgenera (Casimiro-Soriguer et al. 2015). Their presence in both subgenera is consistent with multiple and independent origins of these sexual systems in *Silene.*

In the gynodioecious pathway, the first step is the invasion of a male-sterile mutant in a population with hermaphrodite plants. In the second step, hermaphrodites gradually become functionally male until a female sterile mutant establishes itself, making the population dioecious (Charlesworth and Charlesworth 1978). During this last step, subdioecy occurs because hermaphrodites increase their male fertility but continue to produce some fruits (Bailey and Delph 2007a, Spigler and Ashman 2012). It is suggested that gender plasticity related to environmental conditions may help stabilize a subdioecious population (Delph and

Wolf 2005). With regard to the genetic bases involved in the gynodioecy, in most species the male-sterility alleles necessary to produce female individuals are located in the mitochondria (cytoplasmic male sterility factors) and they interact with nuclear restorers of male fertility (Bailey and Delph 2007b). *Silene vulgaris* and *S. nutans* are examples with multiple cytoplasmic male sterility and nuclear restorer loci involved in the expression of the gynodioecious sexual system (Charlesworth and Laporte 1998, Taylor et al. 2001, Bailey and McCauley 2005, Garraud et al. 2011). The fact that these genes are maternally inherited facilitates the spread of mutants in the population, decreasing the magnitude of female advantage needed to establish females in cosexual populations (reviewed in Dufay and Billard 2012). On the other hand, some models may explain the occurrence of gynomonoecious individuals in gynodioecious-gynomonoecious species by an incomplete male fertility restoration (Koelewijn and van Damme 1996, Ehlers and Thompson 2004). However, there are insufficient experimental studies to test these models (see Garraud et al. 2011).

EVOLUTION OF SEX CHROMOSOMES IN DIOECIOUS SPECIES OF *SILENE*

One of the most fascinating aspects of sexual systems in *Silene* is the acquisition of dioecy in different lineages and possibly at different times (Marais et al. 2011, Slancarova et al. 2013). In the subgenus *Behenantha,* all the species of section *Melandrium* are dioecious (Desfeux et al. 1996, Marais et al. 2011); in the subgenus *Silene*, the group of *S. otites* and relatives includes dioecious and non-dioecious species, although the subsection *Otites* has only dioecious members (Slancarova et al. 2013). According to different authors (Marais et al. 2011, Käfer et al. 2013), dioecy is ancestral in *S. latifolia* and its close dioecious relatives (section *Melandrium*). However, according to Slancarova et al. (2013) it evolved more recently in the group *S. otites* and relatives.

Dioecious species of *Silene* show different sex determination types. Most dioecious species have male heterogamety (e.g. *S. latifolia*), however *S. otites* has female heterogamety, which is very rare in plants and has been reported in only a few angiosperms (reviewed in Slancarova et al. 2013). Moreover, in *S. diclinis*, neo-sex chromosomes have been reported (Weingartner and Delph 2014).

Silene latifolia has X and Y chromosomes (females XX/males XY), whose origin has been suggested in the ancestral lineage of section *Melandrium* (Marais et al. 2011). Compared to other organisms, this is a recent origin (10-20 million years or even much later, Filatov 2005, Slancarova et al. 2013), which allows the study of the early stages of the evolution of sex chromosomes (Ming and Moore 2007, Bergero and Charlesworth 2009, Qiu et al. 2011). It has been suggested that *S. latifolia* sex chromosomes have evolved from a single pair of autosomes through the formation and expansion of a large non-recombining region on the Y chromosome (Filatov 2005, Bergero and Charlesworth 2009). On the other hand, the dioecious *S. diclinis*, included in the same section as *S. latifolia*, has a neo-sex chromosome (a region added to the non-recombining part of a sex-chromosome through a translocation event), which appears to have evolved from the ancestral XY chromosomes present in *S. latifolia* (Weingartner and Delph 2014).

Although Sansome's classic paper (1938) on *S. otites* had already suggested that females were the heterogametic sex, it was not until recently that there have been detailed analyses with molecular markers (Slancarova et al. 2013). They analyzed *S. otites*, showing that females are the heterogametic sex (females ZW/males ZZ). Even more interesting is the fact that *S. colpophylla*, a male heterogametic species, is closely related to *S. otites* and is placed in the same monophyletic group, which supposes an interesting change in heterogamety (Mrackova 2008). On the other hand, the sex-determining system in the

subdioecious *S. roemeri* and *S. acaulis* may have a common origin (Slancarova et al. 2013), although the authors suggest the study of more species to make definite conclusions.

SEX AND GENDER EXPRESSION

Information about sex expression can be gained from simple morphological observations to detailed studies of the genetics and ecology of the species (Sakai and Weller 1999, Elle and Meagher 2000, Delph and Wolf 2005). Changes in sex expression may occur at different levels of organization: (1) in flowers or inflorescences, for example in plants with intra or inter-floral dichogamy (Bertin and Newman 1993, Narbona et al. 2005); (2) in individuals, for example changes in the proportion of male and hermaphroditic flowers in andromonoecious plants (Narbona et al. 2011 and references therein); or (3) in populations, for example sex ratio variations in dioecious populations (Barrett et al. 2010).

Sex expression in *Silene* has been studied in gynomonoecious, gynodioecious-gynomonoecious and dioecious species. For instance, in the gynomonoecious *S. noctiflora,* the frequency of female relative to hermaphroditic flowers within a plant increased due to the effect of high temperature and ethylene (Folke and Delph 1997). In *S. nutans*, the proportion of female flowers in gynomonoecious individuals varied from 0.03 to 0.9 (Dufay et al. 2010). More complexity in sex expression exists when the population level is analyzed. In the dioecious *S. latifolia*, the sex ratio is variable and often female-biased (Carroll and Mulcahy 1993, Austerlitz et al. 2012); in fact, it is affected by environmental variables such as soil moisture and density (Lovett Doust et al. 1987). Even more complexity in sex expression can be found in gynodioecious-gynomonoecious species as a result of the changing frequency of the three possible morphs, as is found in *S. italica* (Maurice 1999), *S. littorea* (Guitián and Medrano 2000, Casimiro-Soriguer et al. 2013), *S. nutans* (Dufay et al. 2010), and *S. stockenii* (Talavera et al. 1996). All these findings suggest that sexual expression in *Silene* species is

plastic, which may have consequences in the maintenance of the sexual system (Pannell et al. 2008, Delph and Wolf 2005).

Sometimes the morphology of the flower does not reflect its function as donor of male or female gametes. A hermaphroditic flower may function as exclusively male or female if its fitness is obtained only through pollen (or seed-siring success) or ovules (or seed production), respectively. Lloyd and Bawa (1984) emphasized consideration not only of the sex of a plant (i.e. morphology), but also the description of gender based on function: its femaleness or maleness as a parent. Plant gender measures allow evolutionary biologists to test hypotheses about sexual system evolution and their environmental relationships (Lloyd 1976, Bawa 1980, Delph and Wolf 2005). Based on the studies of Lloyd (1979, 1980), Lloyd and Bawa (1984) proposed two measurements for plant gender (for clarification, see Barrett and Harder 2006). The phenotypic gender, also called standardized phenotypic gender, quantifies the investment of parental resources (e.g. pollen, seeds, male or female flowers) in relation to other plants of the population. The phenotypic gender of a plant uses estimates of female investment (number of ovules, often estimated as number of female flowers) and male investment (number of pollen grains, male flowers), and includes the equivalence factor (E) which estimates the ratio of investments in maternal and paternal functions in the population as a whole. Values of phenotypic femaleness are defined between zero (a plant that only produces male flowers) and one (only produces female flowers). On the other hand, functional gender estimates the success of a plant as male or female parent, and is calculated as the proportion of a plant's fitness transmitted through ovules or pollen. Accurate estimates of functional gender require information about seed production, pollen availability and dispersal, frequency of self and cross-fertilization of the population, etc. (Lloyd 1980). The maternal expenditure may be easy to measure as seed production, but the estimation of paternal success is a complicated task. The development of molecular tools has facilitated estimation of male

success through paternity analysis (Elle and Meagher 2000, Verdú et al. 2004, Gleiser et al. 2008).

The phenotypic and functional gender of a plant may be similar, but they are not necessarily the same (Primack and Lloyd 1980, Lloyd and Bawa 1984). In fact, study cases showed that both estimates of gender are poorly related, probably due to the complexity of functional gender estimation (Devlin and Stephenson 1987, Méndez 1998, Austen and Weis 2014). In addition, phenotypic gender may also be substantially influenced by the estimates used in its calculation (Thomson and Barrett 1981, Lloyd and Bawa 1984). For instance, the phenotypic gender of the gynodioecious-gynomonoecious *S. littorea* was calculated using two different estimates of female investment: the number of flowers bearing ovules (females and hermaphrodites) and the number of fruits set (Fig. 2, see Casimiro-Soriguer et al. 2013 for

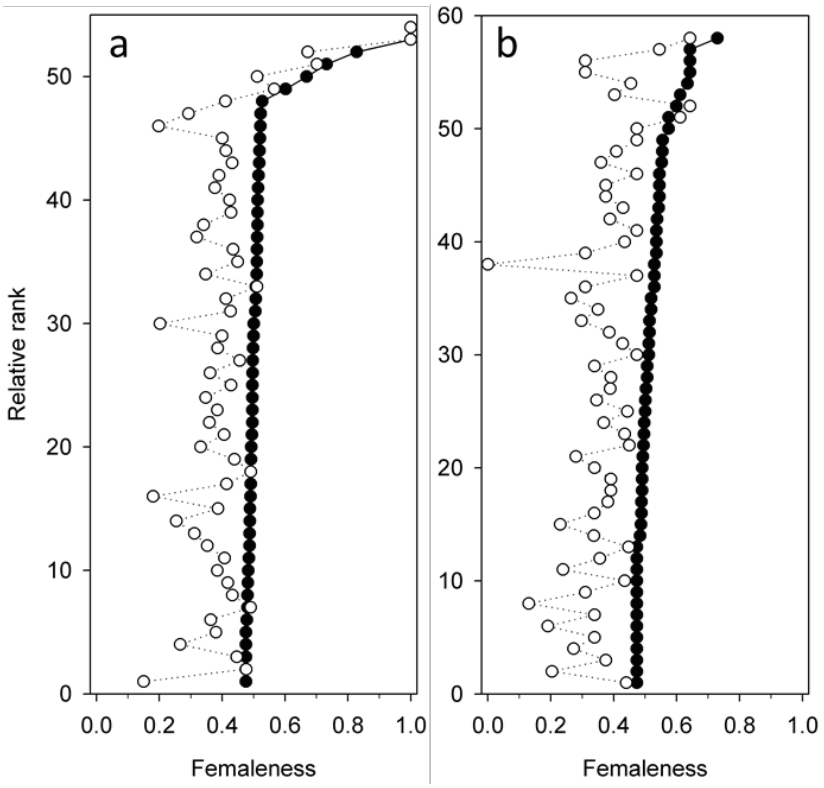

Figure 2. Phenotypic gender in *Silene littorea*. The phenotypic gender has been calculated in two populations (a, b) of *Silene littorea* from southern Spain, using two different estimates of female investment: number of flowers (black circles) and number of fruits (white circles). Plants are ordered by relative value of phenotypic gender calculated by the number of flowers. See Casimiro-Soriguer et al. (2013) for details of the study system.

details of the study system). Phenotypic femaleness estimated with flowers showed that most plants in the populations invested approximately the same in female and male functions (values near 0.5). However, when phenotypic gender was estimated with fruits, there was a decrease in femaleness in most plants of both populations. This means that plants invested more in the male than the female function when fruit estimate was used. It is remarkable that completely female plants (plants that only produced female flowers) of one population showed the same value for both female investment measures (Fig. 2a). However, a completely male plant appeared in the other population when the fruits estimate was used (Fig. 2b); due to the fact that this plant did not produce fruits.

Temporal variation in functional and phenotypic gender of the plants in a population during the flowering season may also occur (Thomson and Barrett 1981, Méndez 1998, Casimiro-Soriguer et al. 2013). The success of a plant as a male may vary over time due to variations in the mating environment, i.e. the amount of pollen (or male-phase flowers) relative to the number of ovules (or female-phase flowers) in the population (Brunet and Charlesworth 1995). Thus, in species with dichogamy and sequential blooming, variations in the mating environment and subsequent variation in individual phenotypic or functional gender are expected (Cruden and Hermann-Parker 1977, Brunet and Charlesworth 1995). Even more complexity can be found when the species present andro(gyno)-monoecious individuals that are able to produce different proportions of male(female) and hermaphroditic flowers. In fact, extremely high fluctuations in mating environment are found in some species with andro(gyno)-monoecious species, dichogamy and synchronous blooming patterns (Thomson and Barrett 1981, Narbona et al. 2011). Although the potential number of species of *Silene* with these characteristics is high, the number of cases studied is limited. In *S. littorea*, the mating environment fluctuated relatively little throughout the flowering season, but fluctuations were higher in a population with low flower production (Casimiro-Soriguer et

al. 2013). This could be important for the conservation of the species since *S. littorea* usually has small populations and presents problems related to inbreeding depression (Vilas et al. 2006).

SEXUAL DIMORPHISM IN SECONDARY SEX CHARACTERS

The term "sexual dimorphism" in plants is used to describe differences between males and females (plants or flowers) in primary and secondary sex characters (Sakai and Weller 1999, Barrett and Hough 2013). Primary sex characters are those that directly refer to the sexual organs (gynoecium or androecium). Secondary sex characters are those related to differences in other structures of the flower (e.g. nectaries, color and size of petals or sepals, or nectar composition, Eckhart 1999), or vegetative traits (e.g. morphology or physiology of vegetative parts, age of first reproduction, longevity and growth; Delph 1999). In this section we will focus on secondary sex characters, differentiating between those affected by reproductive and vegetative traits.

Reproductive traits

In dioecious species, it is expected that evolution drives the differentiation of male and female flowers in response to the different reproductive functions of the two sexes. In fact, many of the differences found between males and females correspond to reproductive characters, particularly flower number and size (Meagher 1992). The prediction based on Bateman's principle (Bateman 1948, Willson 1994) is that males produce larger flowers than females. Contrary to expectations, females of *S. latifolia* have heavier flowers due to sepals and pedicels (Carroll and Delph 1996), although males are able to produce a higher number of flowers than females (Laporte and Delph 1996, Delph et al. 2002), and invest more biomass overall in flowers than females (Carroll and Delph 1996). In this species, the relative number of flowers/flower size has been found to be an important trade-off with other reproductive and

vegetative traits (Delph and Meagher 1995, Delph et al. 2004). Another important aspect analyzed in *S. latifolia* is the quantitative genetic basis of sexual dimorphism (revised in Meagher 1999), and the sex variation in DNA content (more male than female), and its negative correlation to flower size (Meagher and Costich 1994, Meagher et al. 2005). The same significant negative correlation of DNA content and calyx diameter was found in *S. diclinis*, *S. dioica* and *S. latifolia*, but not in *S. marizii* (Meagher and Costich 2004). As in *S. latifolia*, males of the close relative *S. dioica* produced more flowers than females; however, male flowers were larger than females (Kay et al. 1984). On the other hand, Wright and Meagher (2004) found variations between selection in male and female flowers, and this also varied widely between sites and years.

Although the patterns of variation in dioecious species do not always follow the same path, in gynodioecious species the pattern is much clearer, with hermaphroditic flowers with larger corollas than females (Eckhart 1999). This pattern is followed in the gynodioecious species of *Silene*, such as *S. acaulis* (Delph and Carroll 2001) and *S. vulgaris* (Dykstra et al. 2009), but also in the gynodioecious-gynomonoecious species *S. stockenii* (Talavera et al. 1996), *S. nutans* (Dufay et al. 2010), and four other species of the section *Psammophilae* (Casimiro-Soriguer et al. unpublished results).

With respect to sexual dimorphism in nectar production, female flowers of the dioecious *S. latifolia* and *S. dioica* produce higher volumes than males, but sugar concentration is higher in males (Kay et al. 1984, Carroll and Delph 1996, Witt et al. 1999, Gehring et al. 2004). In gynodioecious species in general, the pattern is that hermaphroditic flowers produce more, and more concentrated nectar (Eckhart 1999). In *S. vulgaris* and *S. stockenii*, hermaphroditic flowers had greater sugar content than females (Jolls et al. 1994, Talavera et al. 1996). Females of *S. nutans* produced more nectar than hermaphrodites, although less concentrated (Witt et al. 1999).

Other secondary sex characters such as floral fragrance may have important consequences for reproductive success, via pollinator attraction or florivore deterrence (Schaefer and Ruxton 2011). Studies of dioecious species showed that, in most cases, males emitted more volatiles per flower than females (Ashman 2009). Consequently, despite having smaller flowers than females, floral scent emission has been found to be greater in males of *S. latifolia*, which has important consequences on pollinator behavior (Waelti et al. 2009).

On the other hand, biotic interactions mediated by insect visitors (pollinators, predators or pathogens) may potentially be affected by gender specialization and dimorphism. In several species of *Silene*, a nursery pollination system is found in which noctuids of the genus *Hadena* and *Perizoma* act as pollinators as well as seed predators because they lay eggs on the ovaries of flowers (Jürgens et al. 1996, Kephart et al. 2006, Reynolds et al. 2012). Kephart et al. (2006) found that fruit predation was lower in species with diurnal pollination and hermaphroditic sexual systems compared with nocturnal pollination and dioecious or gynodioecious sexual systems. In this scenario, moths could act as selective agents on sexual systems favoring hermaphroditism. Furthermore, species of *Silene* are frequently infected by the fungal disease *Microbotryum violaceum,* in which pollinators are also potential vectors (Thrall et al. 1993, Hood et al. 2010). This anther-smut pathogen causes plant sterility, producing aborted ovaries and spores instead of pollen. Interestingly, the fungus induces anther development (with fungus spores) in females of dioecious and gynodioecious host species; thus, the fungus is transmitted by male and female plants (Antonovics et al. 2002). Because flower visitors are needed for fertilization and for fungus dispersal, plants may be exposed to conflicting selective forces, attempting to attract pollinators while avoiding fungus transmission (Thrall et al. 1997). Thus, sexual dimorphism in characters related to insect attraction, such as flower number or flower duration, may affect this conflict (Ågren et al. 1999). For example, males of *S. latifolia* and *S. dioca* produce more flowers and are more

frequently visited by insects, and therefore a higher number of spores are deposited in male flowers (Alexander 1989, Shykoff and Bucheli 1995, Carlsson-Graner et al. 1998). Similarly, male flowers of *S. latifolia* have a short flower lifespan compared with females, which reduces the risk of infestation (Kaltz and Shykoff 2001).

Vegetative traits

In long-lived species, males are usually more vigorous than females, and the opposite pattern is found in short-lived species (Barrett and Hough 2013). In *S. latifolia*, males were taller and dedicated more biomass to leaves than females; females had shorter and stouter inflorescences, as a consequence of stopping flower production when fruits started to develop (Gehring and Linhart 1993) and had longer leaves (Delph et al. 2002). In *S. dioica* males had more leaves per rosette, although shorter than females (Cox 1981, Van Nigtevech 1966). In spite of these apparently clear differences, it is important to consider the life history of the plant, because sexual dimorphism before the development of inflorescence seems to be very rare (Zluvova 2010). Significant differences can be found before and after flowering, because reproductive costs can influence future resource distribution (Sánchez-Vilas and Pannell 2011, Barrett and Hough 2013). For example, in *S. latifolia*, Meagher (1992) found that male and female plants had equal seed sizes, early establishment and growth before sexual reproduction. Moreover, in this species, many sexually dimorphic vegetative traits, such as plant height, length of inflorescence branches and allocation of leaves and branches, were found to be correlated with the number of flowers (Gehring and Linhart 1993, Delph et al. 2002, Delph et al. 2005). Purrington and Schmitt (1998) eliminated age differences by sowing seeds of *S. latifolia* on a single day, finding that females emerged earlier but flowered later. A new step in the analysis of dimorphic sex expression is the analysis of sex-specific genes or gene expression. Recently, Zluvova (2010) found three sex-specifically expressed genes in the rosette stage in *S. latifolia*. Lastly, physiological traits also differ between sexes once

flowering has started, but not before. For example, males of *S. latifolia* have higher photosynthetic rates than females (Laporte and Delph 1996), but females live longer than males (Lovett Doust et al. 1987, Carroll and Mulcahy 1993).

CONCLUSIONS AND FUTURE DIRECTIONS

Studies of sexual systems in plants, and specifically *Silene*, are on the increase. In *Silene*, different aspects of sexual system such as the above described, are mostly studied in 14 species, but almost all literature focused on three (*S. latifolia, S. vulgaris* and *S. acaulis*). The dioecious *S. latifolia* has become a model species for the study of sexual dimorphism (Delph and Herlihy 2012, Barrett and Hough 2013), repetitive DNA (Meagher and Vassiliadis 2005) and sex chromosomes in plants (Charlesworth 2013, Slancarova et al. 2013), among other aspects (Bernasconi et al. 2009). In addition, studies of other species have helped to understand new features of sexual systems in plants; for instance, *S. acaulis* has been used to study the role of environmental factors in the transition from gynodioecy to dioecy (Delph 2003), and *S. nutans* for the genetic basis of male sterility in gynodioecy (Garraud et al. 2011).

The three most frequent sexual systems in *Silene* are, in order: hermaphroditism, gynodioecy, and dioecy; but combinations of these three types are also present. This combination of sexual systems in *Silene* makes the genus particularly engaging for the study of evolutionary transitions. A reliable reconstruction of the evolution of sexual systems in *Silene* remains incomplete. This could be due to the difficulties of building accurate phylogenetic relationships among and within the groups of species due to introgression and complex mutational processes (Erixon and Oxelman 2008, Petri and Oxelman 2011), but also to the lack of clear information about the sexual systems of a relevant number of species (Casimiro-Soriguer et al. 2015). Recently, the evolution of dioecy has been clarified in the

two dioecious groups of *Silene* (Marais et al. 2011, Käfer et al. 2013), and gynodioecy seems the most probable pathway for the evolution of dioecy. However, the ancestral sexual system remains unclear (Marais et al. 2011).

Finally, a considerable number of species of *Silene* also showed a variable sexual system within and/or among populations (Jürgens et al. 2002, Casimiro-Soriguer et al. 2015 and references therein), which suggests a plasticity of expression of sexual systems. Particularly interesting are the cases of subdioecious or gynodioecious-gynomonoecious species (Desfeux et al. 1996, Dufay et al. 2010, Casimiro-Soriguer et al. 2013). However, the roles of these sexual systems in the evolution of sexual systems in *Silene* are not yet defined. Experiments designed to clarify the advantage of both sexual systems under different selective scenarios with consideration of functional gender estimates are required.

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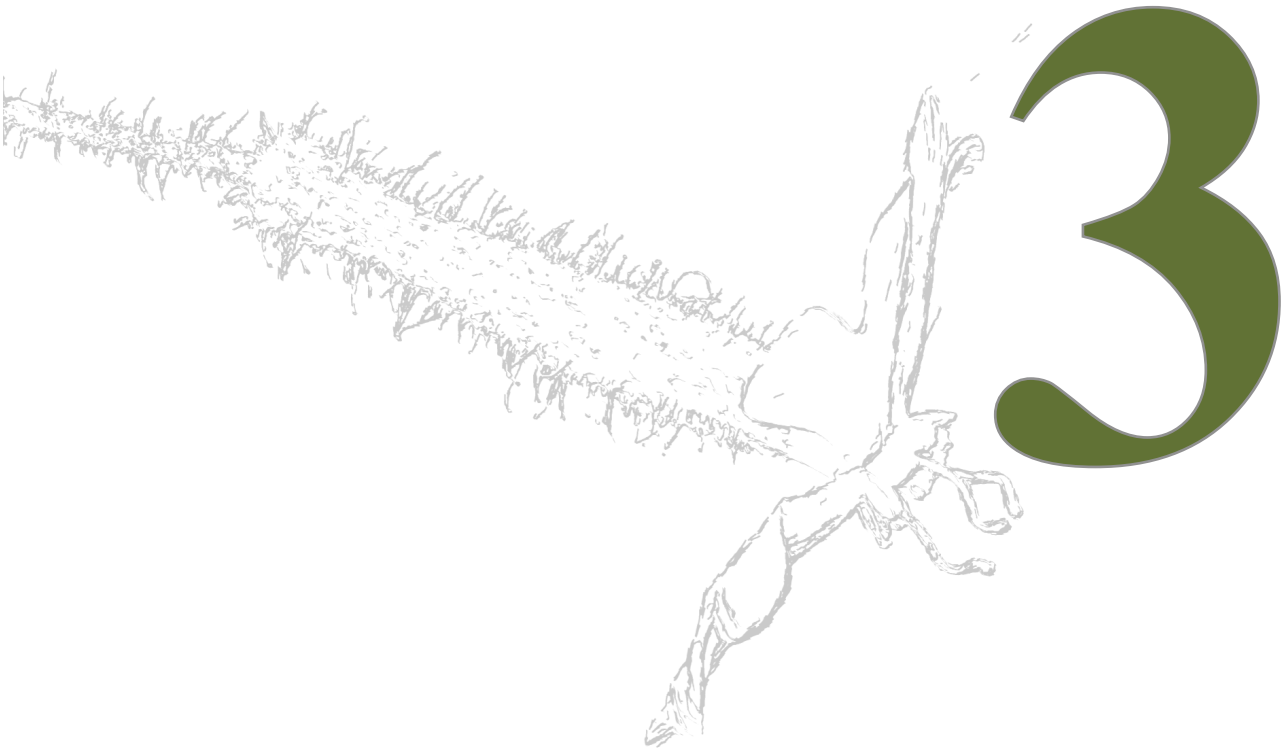

Chapter 3

Diversity of sexual systems within different lineages of the genus *Silene*

Casimiro-Soriguer I, Buide ML, Narbona E (2015) Diversity of sexual systems within different lineages of the genus *Silene*. *AoB Plants* doi: 10.1093/aobpla/plv037.

ABSTRACT

Species and populations can be categorized by their sexual systems, depending on the spatial distribution of female and male reproductive structures within and among plants. Although a high diversity of sexual systems exists in *Silene*, their relative frequency at the genus and infrageneric level is unknown. Here, we carried out an extensive literature search for direct or indirect descriptions of sexual systems in *Silene* species. We found descriptions of sexual systems for 98 *Silene* species, where 63 and 35 correspond to the phylogenetically supported subgenera *Silene* and *Behenantha*, respectively. Hermaphroditism was the commonest sexual system (58.2%), followed by dioecy (14.3%), gynodioecy (13.3%) and gynodioecy-gynomonoecy (i.e. hermaphroditic, female and gynomonoecious plants coexisting in the same population; 12.2%). The presence of these sexual systems in both subgenera suggests their multiple origins. In 17 species, the description of sexual systems varied, and in most cases these differences corresponded to variations within or among populations. Interestingly, the poorly studied gynodioecy-gynomonoecy sexual system showed similar frequency to dioecy and gynodioecy in both subgenera. In addition, the incidence of gynodioecy-gynomonoecy was analysed in the species of section *Psammophilae* (*Silene littorea*, *S. psammitis*, *S. adscendens* and *S. cambessedesii)*, in a survey of 26 populations across the distribution area of the species. The four species showed gynomonoecy-gynodioecy in most populations. Hermaphrodites were the most frequent morph, with a low number of females and gynomonoecious plants in all populations. The frequency of sexual morphs significantly varied among the studied populations but not among species. Female plants generally produced smaller numbers of flowers than hermaphroditic or gynomonoecious plants, and the percentages of female flowers per population were low. All these findings suggest that the gynodioecious-gynomonoecious sexual system in

section *Psammophilae* is closer to hermaphroditism or gynomonoecy than gynodioecy.

KEYWORDS: *Behenantha*; Caryophyllaceae; dioecy; gynodioecy; gynodioecy-gynomonoecy; hermaphroditism; *Psammophilae*; sexual polymorphism; sexual system; *Silene*.

INTRODUCTION

The study of the diversity and evolution of sexual systems in plants has been the focus of many scientists since early days. Species or populations may be categorized by sexual system, depending on the spatial distribution of male and female reproductive structures within and among plants (Bawa and Beach 1981). Although many different sexual systems may exist (Sakai and Weller 1999), most of the angiosperm species belong to one of the five main types: hermaphroditism (72%), gynodioecy (7%, female and hermaphroditic individuals), monoecy, (5%, individuals with female and male flowers) dioecy (5-6%, female and male individuals) and gynomonoecy (3%, individuals with female and hermaphroditic flowers) (Richards 1997, Renner 2014). Although only 5-6% of total angiosperms are dioecious, dioecious species are present in 43% of families, and from 871 to 5000 independent origins of dioecy have been proposed (Renner 2014). Therefore, the evolutionary pathways to dioecy have been the focus of interesting debate, specially the transition from hermaphroditism to dioecy, with gynodioecy or monoecy as intermediate steps (Charlesworth 1999). The association of gynodioecy or monoecy with dioecy at the family or genera level suggests that both are possible pathways to dioecy (Renner and Ricklefs 1995, Dufay et al. 2014, Renner 2014). Gynomonoecy occurs frequently in families such as Compositae or Chenopodiaceae (Yampolsky and Yampolsky 1922, Torices et al. 2011), and has been considered the main route to monoecy from hermaphroditism and vice versa (Torices et al. 2011).

The genus *Silene* (Caryophyllaceae) has been widely used to study the evolution of sexual systems and gender variation (Meagher 2007, Bernasconi et al. 2009, Charlesworth 2013, Weingartner and Delph 2014), and is one of the groups used for the phylogenetic approach (Desfeux et al. 1996, Rautenberg et al. 2010, Marais et al. 2011).

Nonetheless, the complete phylogenetic relationship within this genus is not yet resolved (Rautenberg et al. 2010, Petri et al. 2013). What seems clear is the subdivision of *Silene* (*sensu* Oxelman and Lidén 1995) into two clades: subgenera *Silene* and *Behenantha* (Popp and Oxelman 2004, 2007, Rautenberg et al. 2010). The first of these phylogenetic studies found that dioecy appeared independently at least twice (in subsection *Otites* and section *Melandrium*; according to Oxelman et al. 2013), and that gynodioecy was the most probable ancestral condition for the genus (Desfeux et al. 1996). More recently, Marais et al. (2011) found that either gynodioecy or hermaphroditism could be the ancestral condition of *Silene*. In *Otites* and *Melandrium*, different types of sex-determining systems with a different date of origin are implicated (Käfer et al. 2013, Slancarova et al. 2013). In addition to dioecy, hermaphroditism and gynodioecy are common in *Silene* (Desfeux et al. 1996, Jürgens et al. 2002). However, monoecy is not present, suggesting the evolution of dioecy through the gynodioecy pathway.

Gynomonoecy, andromonoecy (individuals with male and hermaphroditic flowers) and trioecy (populations with hermaphroditic, male and female individuals) have also been reported for *Silene*, but are very rare (Desfeux et al. 1996, Jürgens et al. 2002, present study). However, in a non-negligible number of gynodioecious species, the existence of gynomonoecious individuals (i.e. plants with female and hermaphroditic flowers) in the populations is reported (e.g. Shykoff 1988, Talavera et al. 1996, Lafuma and Maurice 2006, Dufay et al. 2010). Species or populations containing hermaphroditic, female and gynomonoecious individuals must be considered as gynodioecious–gynomonoecious (Gd-Gm hereafter) (Desfeux et al. 1996). The frequency of gynomonoecious plants may be highly variable among populations and species; in some cases, this sexual morph is rare and in others may be the most frequent

(Charlesworth and Laporte 1998, Maurice 1999, Dufay et al. 2010, Casimiro-Soriguer et al. 2013). The genetic mechanism for sex determination of gynodioecy may be based on the interaction of cytoplasmic male sterility genes with nuclear restorers of male fertility (Bailey and Delph 2007), as found in *S. vulgaris* (Charlesworth and Laporte 1998). In some cases, the incomplete restoration of the cytoplasmic male–sterility factors or heteroplasmy (the occurrence of different cytotypes within an individual) can cause partially male-sterile individuals that are able to produce females and hermaphroditic flowers (i.e. gynomonoecious plants) (Koelewijn and Van Damme 1996, McCauley et al. 2005). Thus, although the genetic basis for gynomonoecious and female individuals in Gd-Gm species has been hypothesized in *Silene* species (Glaettli and Goudet 2006, Garraud et al. 2011), their incidence remains unclear.

Silene littorea is one of the most-studied species with a Gd-Gm sexual system (Guitián and Medrano 2000, Vilas and García 2006, Vilas et al. 2006, Casimiro-Soriguer et al. 2013). In several populations from two contrasting sites in their distribution area, the frequency of hermaphrodites or gynomonoecious plants varied highly among populations, but female plants were always rare (Guitián and Medrano 2000, Casimiro-Soriguer et al. 2013). Analysis of functional gender showed nearly all plants in the population transmit their genes via both pollen and ovules; thus, the Gd-Gm sexual system of *S. littorea* seems to be closer to hermaphroditism or gynomonoecy than gynodioecy (Casimiro-Soriguer et al. 2013). Interestingly, *S. stockenii* also shows a Gd-Gm sexual system with a very low frequency of female plants (Talavera et al. 1996). Both species belong to the section *Psammophilae*, composed of three other annual species (*S. adscendens*, *S. cambessedesii* and *S. psammitis*). Therefore, the question which arises from these findings is whether the Gd-Gm sexual system is widespread in the whole *Psammophilae* section. In addition, the reproductive output of

the different morphs may vary in the Gd-Gm sexual system, which may be important to the stable maintenance of these morphs in the populations (Dufay et al. 2010). For instance, Shykoff et al. (2003) found that overall females produce more but smaller flowers, set more fruits and produce more and heavier seeds than hermaphrodites.

In this study, two different approaches were used to evaluate the occurrence of sexual systems, particularly gynodioecy-gynomonoecy, in *Silene*. For the general approach, we searched the literature extensively to locate any direct or indirect description of the sexual system of the species of *Silene*. This search allows us to know the frequency of sexual systems at the genus and infrageneric level as well as their variability within species. Accurate estimates of the frequency of the Gd-Gm sexual system may shed light on their possible evolution and stability in *Silene*, and also in other groups of angiosperms. For the specific approach, we have studied the sexual systems of a total 26 populations of the species of the section *Psammophilae*. Specifically, we seek to answer the following questions: (a) Is the Gd-Gm sexual system widespread throughout the distribution area of *S. littorea* and the other species of section *Psammophilae*?, (b) What is the frequency of the different sexual morphs and types of flowers in the populations?, and (c) Are there differences in the number of flowers produced by each morph?

MATERIALS AND METHODS

Study system

Silene littorea, *S. cambessedesii*, *S. psammitis* and *S. stockenii* are endemic to the Iberian Peninsula and Balearic Islands (Talavera 1979). Talavera (1979) included these taxa together with *S. almolae*, *S. germana* and *S. pendula* within the section *Erectorefractae*. We follow Greuter (1995) who proposed section *Psammophilae*,

previously considered a subsection of *Erectorefractae* (Talavera 1979). Oxelman et al. (2013) considers the species status of *S. adscendens* (previously considered a subspecies of *S. littorea*). All the species are spring–flowering annuals and grow in different types of soil: sandy substrates from the coast (*S. cambessedesii, S. littorea*), dolomites or slates (*S. psammitis*), calcareous sandstones (*S. stockenii*) or schists (*S. adscendens*) (Talavera 1979).

Analysis of the sexual system of section *Psammophilae*

During the peak of the flowering period from 2010 to 2012, we visited five populations of *S. adscendens*, eight of *S. cambessedesii*, eleven of *S. littorea* and four of *S. psammitis* (Fig. 1) [see **Supporting Information**]. We did not include *S. stockenii*

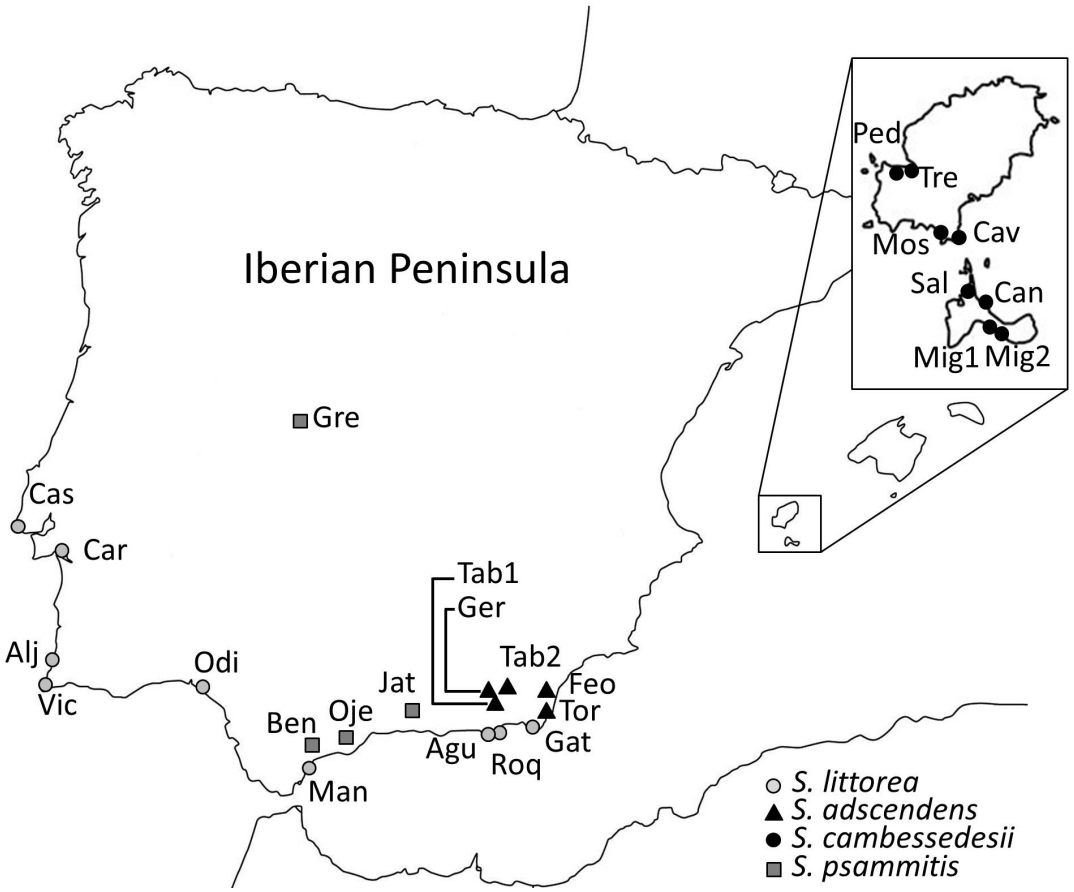

Figure 1. Populations sampled from the different species of section *Psammophilae*: nine populations of *S. littorea* (grey dots), five populations of *S. adscendens* (black triangles), eight populations of *S. cambessedesii* (black dots) and four populations of *S. psammitis* (grey squares).

because: (1) it is a critically endangered species with only a few populations (Bañares et

al. 2004), (2) Talavera et al. (1996) have already studied the sexual system of *S. stockenii* in the most important population, and (3) we visited some of the remnant populations, detecting high florivory levels and a small number of individuals. We performed single–day linear transects of 100 plants, with the exception of some very small populations [see **Supporting Information**]. We chose plants separated by at least 1m to avoid microhabitat or clustering effects in sex expression (Klaas and Olson 2006). For each plant, we counted all the flowers in anthesis, and noted their sex (female or hermaphroditic). Withered flowers were also analysed when sex differentiation was possible. Plants bearing only female or hermaphroditic flowers were considered female or hermaphroditic individuals respectively, whereas individuals with female and hermaphroditic flowers were considered gynomonoecious. In a previous study in *S. littorea*, Casimiro-Soriguer et al. (2013) found that the probability of recording female flowers in gynomonoecious plants was higher when the whole flowering period of a plant was studied than when estimates were based on a single census. Thus, our sampling methodology would underestimate the frequency of gynomonoecious plants in the population. In addition, plants with a large number of flowers would have a higher probability to be classified as gynomonoecious.

Literature search on *Silene* sexual systems

We performed a literature search in the SCOPUS and JSTOR databases including the terms: *Silene*, breeding system, sexual system, hermaphroditic, hermaphrodite, hermaphroditism, dioecious, dioecy, gynodioecious, gynodioecy, gynomonoecious, gynomonoecy, androdioecious, androdioecy, andromonoecious, andromonoecy, monoecious and monoecy. We also revised the description of the *Silene* species in main floras and revision studies or books previous to 1938 that contain information about plant sexuality [see Table 1 and **Supporting Information**]. In

addition, for those species with numerous sexual system descriptions or present in the Euro+Med database, specific individual searches were performed. We annotated the information about the sexual systems of the species in one of the following categories: hermaphrodite (H), dioecious (D), gynodioecious (Gd), gynomonoecious (Gm), androdioecious (Ad), andromonoecious (Am) or trioecious (T). In some cases, mixed sexual systems were found within a single population, for instance Am–Ad (male, hermaphroditic and andromonoecious plants), H–Gm (hermaphrodites and gynomonoecious plants), and gynodioecious–gynomonoecious (Gd-Gm, female, hermaphroditic and gynodioecious plants). When various studies described a species with different sexual systems, all of them were annotated with the respective reference; however, the principal or most frequent sexual system was used for calculating the frequency at the genus or subgenus level (see Jürgens et al. 2002 for similar criteria). The H-Gm category was assigned as H because in most cases gynomonoecious individuals are extremely rare and bear only a few female flowers (e.g. A. Jürgens pers. comm., Giménez-Benavides et al. 2007). We will follow the classification criteria of Oxelman et al. (2013) for *Silene* and the infrageneric level. The subspecies level was not considered.

Statistical analysis

To test for differences in the proportion of the different sexual morphs among species and populations, a generalized linear model (GLM) with a multinomial distribution and a probit link function was carried out. We considered the sexual morph of each individual (female, hermaphrodite or gynomonoecious) as the multinomial response variable; and species and population (nested within species) as fixed factors. Population was treated as a fixed factor rather than a random factor because we are interested to examine differences in morph frequencies among our specific populations,

and the same populations would be analysed in future studies (Bennington and Thayne 1994, Potvin 2001). Comparisons of the number of flowers between female plants and hermaphrodite or gynomonoecious plants were performed using GLMs with a log link function and a Poisson error distribution. The dependent variable was the number of flowers produced by each individual; and sexual morph, population (nested within species) and species were included as fixed factors. On the other hand, the frequency of each sexual system between the subgenus *Silene* and *Behenantha* was compared using X^2 tests for contingency tables (Quinn and Keough 2002). All the analysis were carried out in IBM® SPSS® Statistics v.22.

RESULTS

Sexual system of the section *Psammophilae*

A total of 2478 individuals belonging to 26 populations were surveyed. In general, each studied taxa of section *Psammophilae* showed Gd and Gd-Gm populations, although Gd-Gm populations were the most frequent (Fig. 2). *Silene littorea*, *S. adscendens* and *S. psammitis* showed one Gd population each, whereas *S. cambessedesii* showed two. The remaining populations were all Gd-Gm (Fig. 2).

Overall, the most frequent morph of section *Psammophilae* was the hermaphrodite, with an 86.8% of individuals included in this category, followed by the female and the gynomonoecious morphs (7.9 and 5.3%, respectively). The proportion of hermaphroditic plants within populations ranged from 64.0% (in *S. littorea*) to 99% (in *S. adscendens*), whereas the proportion of female plants varied from 1.0% to 18.0% (in *S. littorea*), and that of gynomonoecious plants ranged from zero (at least one population in each species) to 20% (in *S. psammitis*) (Fig. 2). The frequency of sexual morphs per population significantly varied among the studied populations (Wald X^2 =

62.62, df = 22, P < 0.0001) but not among species (Wald $X^2 = 4.59$, df = 3, P = 0.21).

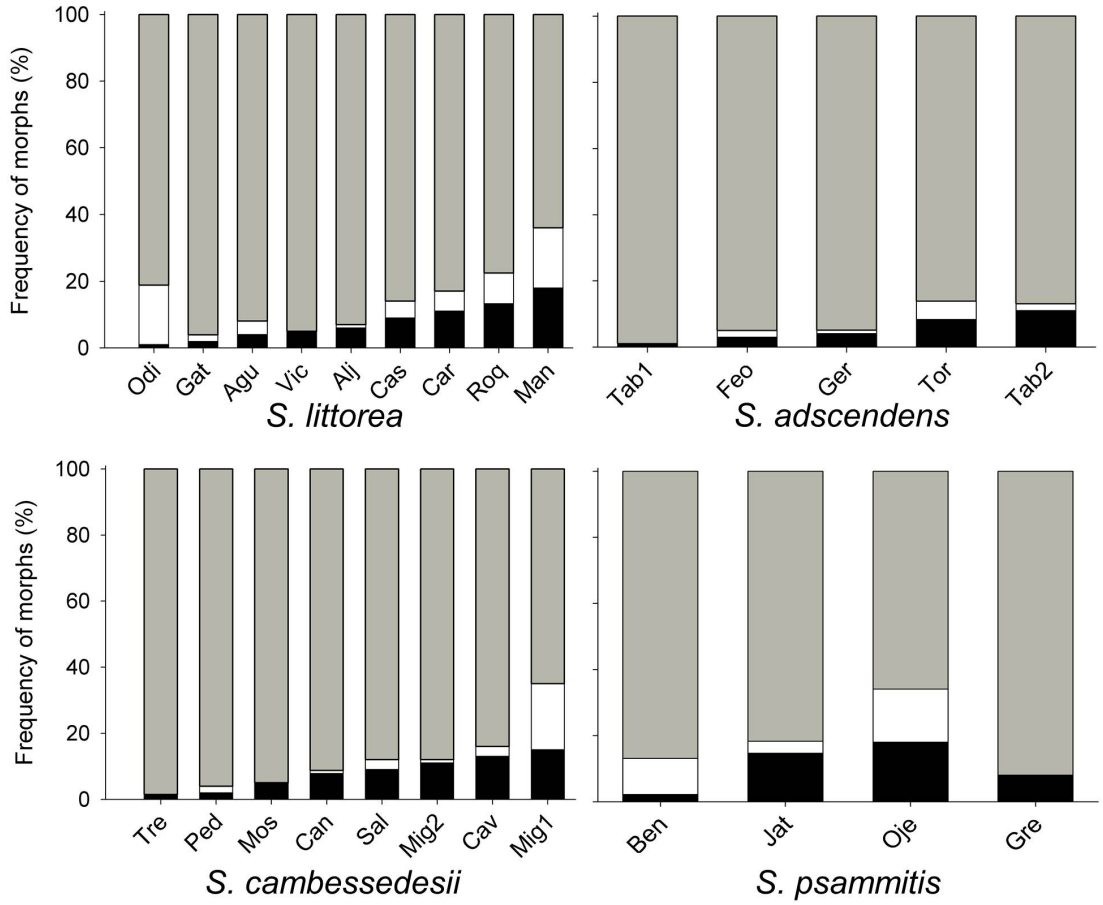

Figure 2. Frequency of hermaphroditic (grey), gynomonoecious (white) and female (black) individuals of species from section *Psammophilae* in each population. The number of individuals per sexual morph sampled in each population is shown in **Supporting Information**.

On the whole, 92.1% of the 7001 flowers analysed were hermaphrodites, and 7.9% were females. At the species level, *S. psammitis* showed the highest proportion of female flowers (16.1%) in the population and *S. adscendens* the lowest (4.8%) [see **Supporting Information]**. The predominance of hermaphrodite flowers was also found at the population level; the mean percentage of female flowers per population ranged from 1.3 to 18.7% in *S. littorea*, from 0.8 to 10.2% in *S. adscendens,* from 1.1 to 14.1% in *S. cambessedesii,* and from 7.1 to 29.4% in *S. psammitis* [see **Supporting Information**]. On the other hand, the percentage of female flowers in gynomonoecious individuals was 34.8% ± 2.0 (mean ± 1SE) in *S. littorea*, 34.5% ± 5.2 in *S. adscendens*,

31.4% ± 3.0 in *S. cambessedesii* and 43.4% ± 3.1 in *S. psammitis* [see **Supporting Information**].

The average number of flowers in female plants was generally smaller than in hermaphroditic or gynomonoecious plants [see **Supporting Information**]. The number of flowers per individual showed significant differences among sex morphs (Wald X^2 = 328.85, df = 2, P < 0.0001), populations (Wald X^2 = 2126.73, df = 22, P < 0.0001) and species (Wald X^2 = 571.88, df = 3, P < 0.0001).

Diversity and frequency of sexual systems in *Silene*

We found that 98 *Silene* species have been specifically studied or described in terms of the sexual system (Table 1). We have collected the data from 46 different species in addition to those formerly found by Desfeux (1996) and Jürgens et al. (2002). The number of species described in the subgenus *Silene* (63 species) is nearly double that in subgenus *Behenantha* (35 species) (Table 1). The most frequent sexual system at the genus level is hermaphroditism (58.2%), followed by dioecy (14.3%), gynodioecy (13.3%) and gynodioecy–gynomonoecy (12.2%). Interestingly, all four sexual systems are present in both subgenera, with a statistically similar frequency (P > 0.43 for all the sexual systems, except for hermaphroditism, that showed marginally significant differences P = 0.09) (Fig. 3). In addition, one Gm and one Am species was found, and both belong to the subgenus *Behenantha.* The fact that 13 of our assigned H species (21.1%) are described as H-Gm in the literature is worthy of mention.

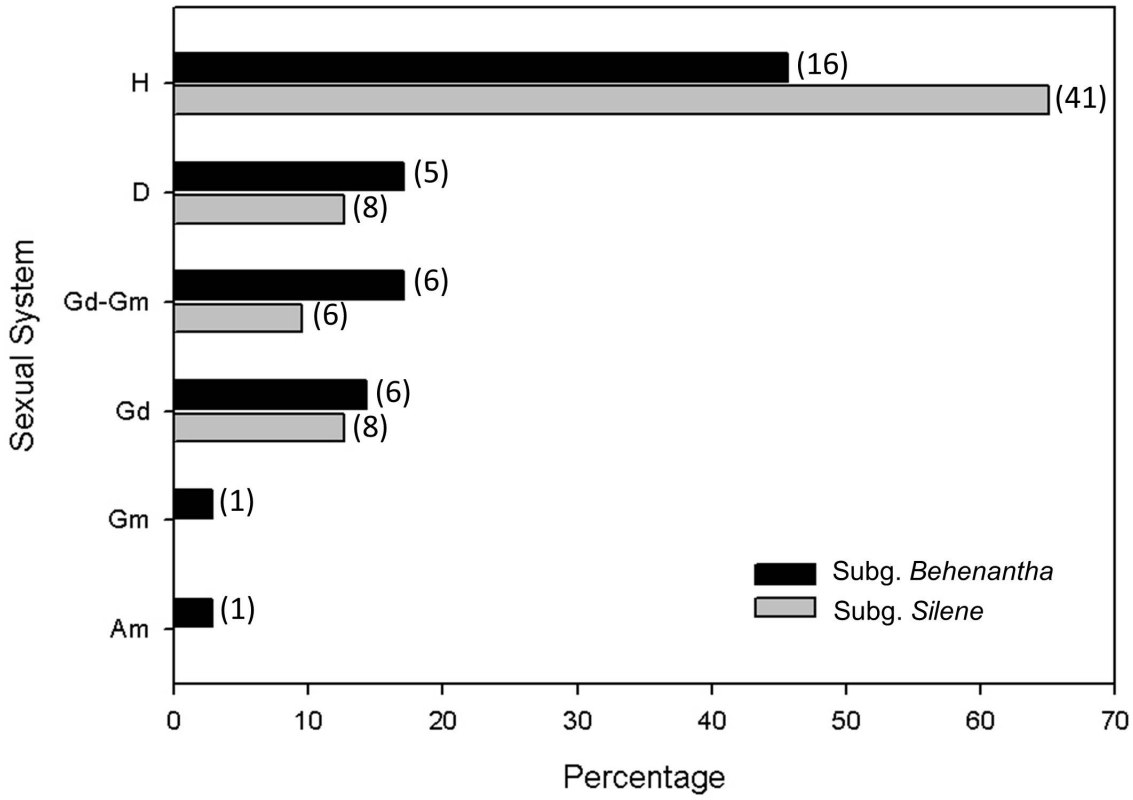

Figure 3. Proportion of sexual systems in subgenus *Behenantha* (black bars) and subgenus *Silene* (grey bars). Abbreviations: Hermaphroditism (H), dioecy (D), gynodioecy-gynomonoecy (Gd-Gm), gynodioecy (Gd), gynomonoecy (Gm) and andromonoecy (Am).

Table 1. Sexual systems in *Silene*. Sexual system description recognizes all the sexual systems described for the species in the literature. The sexual system assigned here is the principal or most frequent sexual system for the species according to our review. Species classification follows Oxelman et al. (2013). Abbreviations: hermaphrodite (H), dioecious (D), gynodioecious (Gd), gynomonoecious (Gm), androdioecious (Ad), andromonoecious (Am) and trioecious (T). Mixed sexual systems are denoted by a dash.

	Sexual system	
Subgenus, section, species	**Described in literature**	**Assigned**
Subgenus *Behenantha* (Otth) Endl.		
Section *Behenantha* Otth		
S. pendula L.	Gd[1,2,51]	Gd
S. uniflora Roth	Gd[3,4,5]	Gd
S. vulgaris (Moench) Garcke	Am-Ad[6]; Gd[2]; Gd-Gm[6,7,8,9,10]	Gd-Gm
Section *Conoimorpha* Otth		
S. conica L.	H[2,7]; H-Gm[2]	H
S. conoidea L.	H[1,2]	H
S. subconica Friv.	Gd[2]	Gd
Section *Dichotomae* (Rohrb.) Chowdhuri		
S. dichotoma Ehrh.	Gd[2,6,7]	Gd
Section *Elisanthe* (Fenzl) Fenzl		
S. noctiflora L.	H[11];H-Gm[2,7];Gm[12,13];Gd-Gm[1]	Gm
Section *Erectorefractae* Chowdhuri		
S. germana Gay	H[51]	H
Section *Melandrium* (Röhl.) Rabeler		
S. astrachanicum (Pacz.) Takht.	D[14]	D
S. diclinis (Lag.) M.Laínz	D[1,8,14,15,16,17]	D
S. dioica (L.) Clairv.	Am[6]; D[2,6,7,8,14,15]	D
S. heuffelii Soó	D[14]	D
S. latifolia Poir.	Am[6]; D[2,6,7,8,14,15]	D
S. marizii Samp.	D[8]	D
Section *Physolychnis* (Bentham) Bocquet		
S. caroliniana Walter	H[18]	H
S. douglasii Hook.	H[11,19]	H
S. gangotriana Pusalkar, D.K.Singh & Lakshmin	H[20]	H
S. laxantha Majumdar	Gd[20,21]	Gd
S. regia Sims	H[22,23]	H
S. rotundifolia Nutt.	H[23]	H
S. scouleri Hook.	H[11]	H
S. stellata (L.) W.T. Aiton	H[18,24]	H
S. tibetica Lidén & Oxelman	H-Am[25]	Am
S. virginica L.	H[18,26]	H
S. zawadzkii Herbich	H[2]	H
Section *Psammophilae* (Talavera) Greuter		
S. adscendens Lag.	Gd-Gm[50]	Gd-Gm
S. cambessedesii Boiss. & Reut.	Gd-Gm[50]	Gd-Gm
S. littorea Brot.	Gd-Gm[2,27,28,50]	Gd-Gm
S. psammitis Link ex Spreng	Gd-Gm[50]	Gd-Gm
S. stockenii Chater	Gd-Gm[29,51]	Gd-Gm

Section *Sedoideae* Oxelman & Greuter		
S. integripetala Bory & Chaub.	H-Gm[30]	H
Section *Viscosae* (Boiss.) C.L.Tang		
S. viscosa (L.) Pers.	H[2,7]	H
Others		
S. acutifolia Link ex Rohrb.	H[8,31]	H
S. elisabethae Jan	H[6,7]	H
Subgenus *Silene*		
Section *Auriculatae* (Boiss.) Schischkin		
S. disticha Willd.	H[2,8]	H
S. echinata Otth	H[8]; H-Gm[2]	H
S. linicola C.C.Gmel.	H[2,6,8]	H
S. schafta J.G.Gmel. ex Hohen.	Gd[2]	Gd
S. spergulifolia (Willd.) M.Bieb.	H[2]	H
S. vallesia L.	Gd[2,7]	Gd
Section *Silene*		
S. apetala Willd.	H[1,2]	H
S. ciliata Pourr.	H-Gm[32]	H
S. colorata Poir.	H[8,33]; H-Gm[2]	H
S. gallica L.	H[8]; H-Gm[2]	H
S. gracilis DC.	H[51]	H
S. micropetala Lag.	H[8]; H-Gm[2]	H
S. nicaeensis All.	H[2,8,51]	H
S. nocturna L.	H[1,2,8]	H
S. pseudoatocion Desf.	Gd-Gm[2]	Gd-Gm
S. ramosissima Desf.	H[8,51]	H
S. scabriflora Brot.	H[51]	H
S. secundiflora Otth	H[2,8]	H
S. sericea All.	Gd[2]	Gd
S. succulenta Forssk.	H[2]	H
Section *Siphonomorpha* Otth		
S. acaulis (L.) Jacq.	D[2,6,7,14]; T[6,7,15,34]; Gd-Gm[35]	D
S. andryalifolia Pomel	Gd[2]	Gd
S. borysthenica (Gruner) Walters	D[15,36]	D
S. colpophylla Wrigley	D[37]	D
S. cyri Schischkin	D[14]	D
S. fernandezii Jeanm.	H-Gm[8]	H
S. flavescens Waldst. & Kit.	H-Gm[2]	H
S. fruticosa L.	H[2]	H
S. gazulensis Galán,Cortés, Orell. & Morales Alonso	H[38]	H
S. gigantea L.	H-Gm[39]	H
S. hayekiana Hand.-Mazz. & Janch.	Gd[2]	Gd
S. hellmannii Claus	D[14,15]	D
S. hifacensis Rouy	Gd[23]; Gd-Gm[40]	Gd-Gm
S. italica (L.) Pers.	H[7]; Gd[2]; Gd-Gm[1,41,42]	Gd-Gm
S. multicaulis Guss.	H[2]	H
S. multiflora (Ehrh) Pers.	H[7]	H
S. nocteolens Webb &	H[38]	H

Berthel.		
S. nutans L.	Am-Ad[6]; Gd-Gm[1,2,6,7,43]	Gd-Gm
S. otites (L.) Wibel	Ad[6]; D[1,2,6,7,15,36]	D
S. paradoxa L.	H[2,23]	H
S. parnassica Boiss. & Spruner	H[2]	H
S. patula Desf.	H[44]	H
S. roemeri Friv.	Gd-Am[15]; H-Gd[2]	Gd
S. saxifraga L.	Am-Gm-T[6]; Gm[1]; Gd-Gm[2,7]	Gd-Gm
S. sendtneri Boiss.	D[15]	D
S. senneni Pau	H[45]	H
S. thessalonica Boiss. & Heldr.	H-Gm[2]	H
S. viridiflora L.	H[7]; Gd-Gm[2]	Gd-Gm
S. waldsteinii Griseb.	H-Gm[2]	H
S. wolgensis (Hornem.) Otth	D[36]	D
Section *Spergulifoliae* (Boiss.) Schischkin		
S. brahuica Boiss.	Gd[21]	Gd
Others		
S. bupleuroides L.	H[2,7]	H
S. capitellata Boiss.	H[46]	H
S. chlorantha (Willd.) Ehrh	H[2,7,47]	H
S. cretica L.	H[2,7,8]	H
S. friwaldskyana Hampe.	H[2]	H
S. hawaiiensis Sherff	H[23,48]	H
S. inaperta L.	H[1,2]	H
S. isaurica Contandr. & Quézel	Gd[46]	Gd
S. kemoniana C. Brullo, Brullo, Giusso, Ilardi & Sciandr.	H[49]	H
S. muscipula L.	H[2]	H
S. portensis L.	H[2,51]	H
S. struthioloides A.Gray	H[23,48]	H

[1]Desfeux et al. 1996, [2]Jürgens et al. 2002, [3]Baker and Dalby 1980, [4]Pettersson 1997, [5]Warren and James 2008, [6]Knuth 1908, [7]Meusel and Mühlberg 1979; [8]Talavera 1990, [9]Glaettli and Goudet 2006, [10]Miyake and Olson 2009, [11]Touzet and Delph 2009, [12]Folke and Delph 1997, [13]Davis and Delph 2005, [14]Schischkin 1970, [15]Chater and Walters 1964, [16]Prentice 1976, [17]Montesinos et al. 2006, [18]Reynolds et al. 2009, [19]Kephart et al. 1999, [20]Pusalkar et al. 2004, [21]Tropicos.org, [22]Dolan 1994, [23]Moyle 2006, [24]Castillo et al. 2013, [25]Oxelman et al. 2001, [26]Dudash and Fenster 2001, [27]Guitian and Medrano 2000, [28]Casimiro-Soriguer et al. 2013, [29]Talavera et al. 1996, [30]Oxelman 1995, [31]Buide and Guitián 2002, [32]Giménez-Benavides et al. 2007, [33]Terrab et al. 2007, [34]Alatalo and Molau 2001, [35]Shykoff 1992, [36]Lihua et al. 2001, [37]Mrackova et al. 2008, [38]Bañares et al. 2004, [39]Ghazanfar 1989, [40]Prentice et al. 2003, [41]Maurice 1999, [42]Lafuma and Maurice 2006, [43]Dufay et al. 2010, [44]Naciri et al. 2010, [45]Martinell et al. 2010, [46]Yildiz and Çirpici 2013, [47]Lauterbach et al. 2011, [48]Westerberg and Saura 1994, [49]Brullo et al. 2012, [50]Casimiro-Soriguer et al. present study, [51]E.Narbona, M.L. Buide and I. Casimiro-Soriguer, personal observations.

There are some sections whose species present mainly the same sexual system. For instance, section *Melandrium* are all dioecious, section *Psammophilae* are all gynodioecious-gynomonoecious, and section *Physolychnis* are all hermaphroditic except one species (*S. laxantha,* Table 1). Other sections seem more variable. Thus, section *Silene* includes H, Gd and Gd-Gm species, and section *Siphonomorpha* includes all the sexual systems present in the subgenus; however, it can be said that both sections have the highest number of species with known sexual systems.

In the literature, we have found 17 species (17.3%) whose description of the sexual system varies. For instance, *S. noctiflora* has been described as H, H-Gm, Gm, or Gd-Gm, and *S. acaulis* as D, T or Gd-Gm. By contrast, in other species, the sexual system description is consistently confirmed by several studies (e.g. the dioecious *S. diclinis* or the hermaphroditic *S. chloranta*).

DISCUSSION

Studies of sexual systems over an entire section of *Silene* are mostly focused on those groups containing dioecious species (Marais et al. 2011, Slancarova et al. 2013). To the best of our knowledge, this is the first study that analyses the sexual system of a whole section in *Silene* composed of non–dioecious species, including multiple populations across their distribution area. All species of section *Psammophilae* should be considered Gd-Gm, despite their traditional description as hermaphrodites (Talavera 1990, Greuter 1995). The Gd-Gm sexual system has been described in other species of the Caryophyllaceae family (e.g. *Dianthus sylvestris*, *Gypsophila repens, Stellaria longipes*; Philipp 1980, Collin and Shykoff 2003, Lopez-Villavicencio et al. 2005). However, their presence in other families seems scarce; only a few cases are known in the Plantaginaceae and Lamiaceae (Khey-pour 1980, Koelewijn and Van Damme 1996,

Widén and Widén 1999).

We have found that hermaphroditic plants are the most frequent morph in all populations and species, including other species in the section *Psammophilae*, *S. stockenii* (Talavera et al. 1996). Similar results have been found in other Gd-Gm species of *Silene*, such as *S. italica* and *S. nutans* (Maurice 1999, Dufay et al. 2010). We also have demonstrated that the proportion of gynomonoecious plants varies among populations. Other previously studied populations of *S. littorea* showed hermaphroditic individuals in a similar or smaller frequency than the gynomonoecious (Guitián and Medrano 2000, Casimiro-Soriguer et al. 2013). This fact may be explained by this high inter-population variability in the frequency of hermaphroditic plants, but variations due to the different sampling methodology cannot be excluded. At least in *S. littorea* it is possible that plants classified as hermaphrodites in a single-census day could produce a female flower throughout the flowering period (Casimiro-Soriguer et al. 2013).

Most *Silene* species reviewed here were hermaphroditic (ca. 60%), but dioecious, gynodioecious, and Gd-Gm species were also relatively frequent. Interestingly, this genus was considered predominantly gynodioecious by some authors (Matsunaga and Kawano 2001, Lengerova et al. 2003, Slancarova et al. 2013). Knuth (1908) reported the presence of androdioecy or andromonoecy in several species, but this has never been confirmed in further studies. More recently, Oxelman et al. (2001) described the presence of apparently functionally male flowers in the lateral positions of the dichasium in *S. tibetica*, but again no further studies have confirmed this finding. On the other hand, hermaphroditism, dioecy, gynodioecy and Gd-Gm are present in both subgenus *Behenantha* and *Silene* at similar frequencies. The presence of each sexual system in both phylogenetically supported subgenera suggests a repeated independent evolution of sexual systems in these *Silene* clades, as found in other groups (Renner and

Won 2001, Soza et al. 2012). In fact, repeated evolution of dioecy is phylogenetically confirmed in *Silene* (Marais et al. 2011, Slancarova et al. (2013).

Our survey of sexual systems in *Silene* showed that although most species seem to be consistent in their sexual system, 17% of the reported species were described with more than one sexual system. This variation may be caused by different authors assigning sexual systems (e.g. *S. dioica* and *S. latifolia* are dioecious, but have been considered andromonoecious by Knuth (1908)) or by authors' simplification due to the low frequency of some sexual morphs in populations. However, in most cases these differences could correspond to variations within or among populations (e.g. *S. acaulis, S. noctiflora, S. saxifraga* and *S. vulgaris*; see references in Table 1). This variation may be related to the genetic basis of sex determination and/or ecological factors acting on sexual expression (Delph 2003, McCauley and Bailey 2009). For example, the sexually plastic *S. acaulis* shows dioecy, trioecy, gynomonoecy or gynodioecy across its distribution area (Maurice et al. 1999, Alatalo and Molau 2001, Delph and Carroll 2001) and a cytoplasmic determination of sex with nuclear male fertility restorer genes is suggested (Delph et al. 1999, Klaas and Olson 2006). In addition, the role of environmental factors in sex expression has also been demonstrated in different species with higher frequency of female plants in harsher or dryer environments (Delph 2003). For instance, a higher female frequency in low-quality sites was found in *S. acaulis* (Delph and Carroll 2001).

A question arising from the relative high frequency of gynodioecy–gynomonoecy in *Silene*, and particularly in section *Psammophilae*, is whether this sexual system is an evolutionarily stable strategy. Theoretical models suggest that gynodioecy can evolve into dioecy, but also can be stable (Charlesworth and Charlesworth 1978, Dufay et al. 2014). Less is known about the maintenance of

gynomonoecy (De Jong et al. 2008, Mamut et al. 2014), and especially Gd–Gm (McCauley and Bailey 2009, Garraud et al. 2011). In an evolutionarily stable Gd–Gm sexual system, female and gynomonoecious individuals must compensate for their loss of male function at the individual and flower level, respectively (Lloyd 1984). In gynodioecy, the advantage of female plants over hermaphrodites can be through inbreeding avoidance, resource reallocation or sex-difference interactions with herbivores (Ashman 2002, Dufay and Billard 2012). The degree of female advantage should have an impact on the frequency of females (Dufay et al. 2007). In that case, those species with low female advantage will have low or variable frequency of female plants (Dufay et al. 2014). We found a low frequency of female plants per population in all species of section *Psammophilae*, as well as in the Gd-Gm species *S. italica* and *S. nutans* (Maurice 1999, Dufay et al. 2010). In these species, female advantage over hermaphrodites due to reallocation of resources seems to be low (Lafuma and Maurice 2006, Dufay et al. 2010). For instance, in *S. nutans* there were no differences in seed mass, germination rate or offspring quality between females and hermaphrodites (Dufay et al. 2010). In *S. stockenii*, females produced similar fruit set and number of seeds to hermaphroditic or gynomonoecious plants (Talavera et al. 1996). Similarly, in *S. littorea* female plants set similar fruits than gynomonoecious or hermaphrodites plants (Guitián and Medrano 2000). We have found that the number of flowers in female plants was smaller than in the other morphs in some of the populations analysed. Thus reproductive output of female plants in the section *Psammophilae* seems to be lower than those of gynomonoecious or hermaphrodites, but further studies are needed to assess the possible female advantage in these species. On the other hand, the avoidance of inbreeding depression by female plants of *S. littorea* could help to maintain this morph in the population, although in a low frequency (Vilas and García 2006).

With regard to reproductive compensation of gynomonoecious plants over hermaphrodites, three main hypotheses have been proposed: (i) two types of flowers may allow the reallocation of resources to male and female functions (Lloyd 1979), (ii) female flowers can partially avoid inbreeding depression by favouring outcrossing (Marshall and Abbott 1984, Mamut et al. 2014), and (iii) flowers can escape florivory since hermaphrodites are usually more often attacked (Ashman 2002, Bertin et al. 2010). The outcrossing-benefit hypothesis of gynomonoecy has been demonstrated in *Eremurus anisopterus* (Mamut et al. 2014) and in *S. noctiflora* (Davis and Delph 2005). In the former, perfect flowers promote seed quantity by increasing pollinator attraction, whereas in the latter perfect flowers provide reproductive assurance by autonomous selfing when pollinators are scarce. As *Silene* species are self–compatible, autogamous selfing is possible where there is an overlap between sexual phases in the protandrous hermaphroditic flowers (Davis and Delph 2005, M.L. Buide unpubl. data). In three populations of *S. littorea,* around 20% of seed set was due to autonomous selfing (Hidalgo-Triana 2010), with similar findings for *S. stockenii* (23%; Talavera et al. 1996). Thus, in these species of section *Psammophilae*, perfect flowers in gynomonoecious plants could allow some levels of reproductive assurance, whereas female flowers could partially avoid inbreeding depression. On the other hand, environmental factors could also affect the production of female flowers in gynomonoecious plants, and consequently affect sex expression in species of section *Psammophilae*. In the gynomonoecious *S. noctiflora*, an increase of 6 ºC in a greenhouse, increased the production of female flowers in gynomonoecious plants (Folke and Delph 1997).

CONCLUSIONS

To sum up, we have confirmed the high diversity of sexual systems in *Silene,* but we have also demonstrated that the most important sexual systems are similarly represented in both subgenera *Silene* and *Behenantha*. The Gd-Gm sexual system is found in a similar number of species as dioecy and gynodioecy. In addition, we have documented that most populations of species from section *Psammophilae* showed a Gd-Gm sexual system, but variations in sexual expression also exist. The low number of females and gynomonoecious plants, and the low percentage of female flowers at the population level, suggest that the Gd-Gm sexual system in section *Psammophilae* is closer to hermaphroditism or gynomonoecy than gynodioecy. Thus, our study generates an important question: Has the Gd–Gm sexual system any advantage over hermaphroditism and gynodioecy, or is it just a consequence of the genetic mechanism of gynodioecious sex determination? The main non–exclusive hypotheses proposed for the determination of the gynomonoecious morph are the effect of environmental factors, and the partial restoration of male fertility (Dufay et al. 2010 and references therein). However, to the best of our knowledge, explicit evolutionary models do not exist including the gynomonoecious plants and their role on evolutionary transitions (Garraud et al. 2011). Gd-Gm species of *Silene,* and especially those of the section *Psammophilae,* could be a good model system to study the maintenance of gynomonoecious individuals in Gd-Gm populations.

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CONTRIBUTIONS BY THE AUTHORS

EN, MLB and ICS conceived the idea and collect the field data. ICS performed the literature review. EN and ICS ran the statistics. ICS led the writing with assistance of the others.

CONFLICTS OF INTEREST STATEMENT

No conflicts of interest.

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SUPPORTING INFORMATION

File S1. Geographic coordinates and number of individuals analysed (N) in populations of species from section *Psammophilae*.

Taxon	Country	Population	Code	Latitude (°N)	Longitude (°E)	N
S. littorea	Spain	Cabo de Gata	Gat	36.765550	-2.230917	102
		Roquetas de Mar	Roq	36.713056	-2.635889	98
		San Agustin	Agu	36.691028	-2.701528	100
		Manilva	Man	36.332278	-5.239083	100
		Odiel	Odi	37.164706	-6.919111	101
	Portugal	Cabo San Vicente	Vic	37.026394	-8.991392	100
		Aljezur	Alj	37.338767	-8.851828	100
		Cascais	Cas	38.702153	-9.473942	100
		Carrasqueira	Car	38.400822	-8.710925	100
S. adscendens	Spain	Gergal	Ger	37.083361	-2.507861	99
		Cala Los Toros	Tor	36.822639	-2.043222	72
		Los Feos	Feo	37.013444	-2.029278	100
		Tabernas1	Tab1	37.007508	-2.456094	100
		Tabernas2	Tab2	37.115250	-2.404389	100
S. cambessedesii	Spain	Platja des Cavallet	Cav	38.848139	1.401056	100
		Can Mosson	Mos	38.870306	1.347972	60
		Punta des Trencs	Tre	38.969194	1.270722	68
		Punta sa Pedrera	Ped	38.970028	1.261111	101
		Ses Salines	Sal	38.746806	1.432889	100
		Platja ses Canyes	Can	38.729528	1.451861	103
		Platja Migjorn1	Mig1	38.684389	1.467500	100
		Platja Migjorn2	Mig2	38.680806	1.481972	100
S. psammitis	Spain	Jatar	Jat	36.916194	-3.905028	82
		Benahavís	Ben	36.511000	-5.035750	92
		Ojén	Oje	36.592972	-4.857389	100
		Sierra de Gredos	Gre	40.215608	-5.247733	100

File S2. Revised literature that was not cited in the manuscript because no information about the sexual system of species was found.

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File S3. Percentage of female flowers (FF) in populations, average number of flowers per sexual morph and percentage of female flowers in gynomonoecious (GM) plants. Mean ± s.e. per species is highlighted in bold.

			Number of flowers per morph						% FF in	
		% FF per	**Females**		**Hermaphrodites**		**Gynomonoecious**		GM plants	
SPECIES	POP	population	Mean (N)	SE	Mean (N)	SE	Mean (N)	SE	Mean	SE
	Roq	10.4	1.7 (13)	0.4	4.4 (76)	0.7	7.7 (9)	2.3	32.0	6.3
	Gat	1.3	1.0 (2)	0.0	3.0 (98)	0.3	5.5 (2)	1.5	19.6	5.4
	Odi	5.9	4.0 (1)	-	5.3 (82)	0.4	4.7 (18)	0.7	37.3	3.9
	Vic	2.1	1.2 (5)	0.2	3.0 (95)	0.4	-	-	-	-
S littorea	Alj	2.2	3.7 (6)	1.3	12.0 (93)	1.6	7.0 (1)	-	42.9	-
	Cas	10.6	1.8 (9)	0.7	2.0 (86)	0.2	3.4 (5)	0.8	41.7	9.1
	Agu	4.2	1.8 (4)	0.5	2.9 (92)	0.2	5.0 (4)	0.7	26.7	-
	Man	18.7	1.8 (18)	0.3	3.0 (64)	0.3	5.1 (18)	1.2	36.4	-
	Car	12.9	2.3 (11)	0.5	2.3 (83)	0.2	4.0 (6)	0.9	30.4	5.3
	Mean ± s.e.	**7.6 ± 2.0**	**2.0**	**0.2**	**4.3**	**0.3**	**5.14**	**0.5**	**34.8**	**2.0**
	Ger	3.2	1.0 (4)	0.0	1.6 (94)	0.1	2.0 (1)	-	50.0	-
	Tab1	7.1	1.0 (1)	-	1.3 (99)	0.1	-	-	-	-
S. adscendens	Tab2	0.8	2.4 (11)	0.8	4.2 (87)	0.3	2.0 (2)	0.0	50.0	0.0
	Feo	2.9	1.3 (3)	0.3	2.0 (95)	0.1	5.5 (2)	2.5	22.9	10.4
	Tor	10.2	1.3 (6)	0.2	1.4 (62)	0.1	4.0 (4)	0.7	28.8	7.2
	Mean ± s.e.	**4.8 ± 1.7**	**1.7**	**0.4**	**2.1**	**0.1**	**3.7**	**0.7**	**34.5**	**5.2**
	Tre	1.1	1.0 (1)	-	1.3 (67)	0.1	-	-	-	-
	Ped	2.3	1.5 (2)	0.5	2.1 (97)	0.2	3.5 (2)	0.5	29.2	4.2
	Sal	9.8	1.3 (9)	0.2	1.5 (88)	0.1	2.3 (3)	0.3	44.4	5.6
	Can	8.0	1.1 (8)	0.1	1.1 (94)	0.0	2.0 (1)	-	50.0	-
S. cambessedesii	Mig1	14.1	1.9 (15)	0.5	3.0 (65)	0.4	14.1 (20)	3.7	26.6	3.7
	Mig2	10.2	1.0 (11)	0.0	1.2 (88)	0.1	2.0 (1)	-	50.0	-
	Cav	11.1	1.0 (13)	0.0	1.4 (84)	0.1	3.3 (3)	1.3	38.9	11.1
	Mos	3.6	1.0 (3)	0.0	1.4 (57)	0.1	-	-	-	-
	Mean ± s.e.	**7.5 ± 1.7**	**1.3**	**0.1**	**1.6**	**0.1**	**10.3**	**2.7**	**31.4**	**3.0**
	Jat	18.6	2.5 (12)	0.6	2.2 (67)	0.2	3.0 (3)	0.6	44.4	5.6
	Ben	7.1	1.0 (2)	0.0	2.4 (80)	0.2	4.7 (10)	0.8	36.6	5.0
S. psammitis	Oje	29.4	2.2 (18)	0.3	1.9 (66)	0.1	3.3 (16)	0.4	47.5	4.4
	Gre	9.5	2.4 (8)	0.5	2.0 (92)	0.1	-	-	-	-
	Mean ± s.e.	**16.1 ± 5.1**	**2.3**	**0.3**	**2.1**	**0.1**	**3.8**	**0.4**	**43.4**	**3.1**

4

Chapter 4

Flower dimorphism and selection on flower size in four gynodioecious-gynomonoecious species *of Silene*

Casimiro-Soriguer I, Narbona E, Buide ML, Arista M. Flower dimorphism and selection on flower size in four gynodioecious-gynomonoecious species of *Silene* (manuscript).

ABSTRACT

Sexual dimorphism in gynodioecious species exhibits an homogeneous pattern, with hermaphroditic flowers usually larger than females. This could be the result of a more intense sexual selection on the morph carrying the male function, because male reproductive success depends more on access to mating opportunities, whereas female function is more limited by resources. In this study we investigate the possibility of negative selection on female individuals by pollinators in four gynodioecious-gynomonoecious species of *Silene* section *Psammophilae* (*S. adscendens*, *S. cambessedesii*, *S. littorea* and *S. psammitis*) accounting for inter-population differences. We have measured several floral traits and both pollen deposition and germination on stigmas of female and hermaphroditic flowers. We then performed phenotypic selection analyses to know if pollinators preferred hermaphroditic flowers over females due to differences in size in several populations. We found that hermaphroditic flowers are larger than female flowers in all the studied species, and produce more ovules except in *S. psammitis*. Pollen deposition was higher in hermaphroditic than female flowers; however, pollen germination in female and hermaphroditic flowers was statistically similar in most species. Selection gradients for flower size through both pollen deposition and germination were mostly positive for all populations, indicating directional selection to larger flowers, however only some populations showed significant selection gradients. If we assume that selection on larger flowers indicates selection on hermaphroditic flowers, then female flowers (and consequently) female individuals could be suffering from pollen limitation, which could difficult the maintenance of females in populations unless other factors, such as avoidance of inbreeding depression, are acting.

INTRODUCTION

Understanding the mechanisms which maintain the diverse sexual strategies of plants has remained an important challenge for evolutionary biologists. In some species, populations are sexually polymorphic and contain two or more sex phenotypes. One of the most common sexual polymorphisms in flowering plants is gynodioecy (Webb 1999), which consists of the coexistence of females and hermaphrodites within populations of the same species. In gynodioecious species, the effect of natural selection, random processes and gene flow on the maintenance of sex polymorphism is still open to debate (De Cauwer et al. 2012).

Evolution from hermaphroditism to dioecy requires an intermediate step, where gynodioecy and monoecy have been proposed as the most common pathways (Dufay et al. 2014, Renner et al. 2014). Gynodioecy is present in more than 18% of angiosperm families, although in less than 2% of genera (Dufay et al. 2014). In most gynodioecious species, male-sterility is determined by a mitochondrial gene, while male function can be restored by a nuclear gene; purely nuclear control of sex is less common (Budar and Pelletier 2001). For gynodioecy to persist in populations, theoretical models consider that female plants need a reproductive advantage over hermaphrodites to compensate for the loss of male function (Bailey et al. 2003). Females can produce more or better seeds than hermaphrodites due to reduced inbreeding depression, reallocation of resources from male to female function, and sex-differences in interactions with herbivores (Ashman 2002, Dufay and Billard 2012). To maintain gynodioecy, the degree of female advantage depends on the placement of male-sterility mutations. If they are in the nucleus, the female advantage needs to be twice that of hermaphrodites, whereas if male-sterility mutations are in the cytoplasm, the advantage needs to be slightly higher (Charlesworth and Charlesworth 1978, Bailey et al. 2003, Bailey and

Delph 2007). The frequency of females in populations can be associated with the degree of that female advantage; therefore, when female advantage over hermaphrodites is great, female frequency tends to be high (Dufay and Billard 2012).

In gynodioecious species, reproduction depends to a greater or lesser extent on pollinator attendance. Floral dimorphism between female and hermaphroditic flowers is a common aspect of those species, with hermaphroditic flowers commonly larger (Darwin 1877, Baker 1948, Delph 1996, Eckhart 1999, Shykoff et al. 2003). In general, male reproductive function depends on the availability of pollinators to a higher degree than female function (e.g. Queller 1983, Willson and Burley 1983, Stanton et al. 1986). In gynodioecious species, the larger size of hermaphrodite flowers could be the result of a more intense sexual selection on the morph carrying the male function, whose reproductive success depends more on access to mating opportunities, whereas female function is more limited by resources (Bateman 1948).

In entomophilous species, pollinators are considered one of the main forces of selection (Fenster et al. 2004), although antagonists (e.g. herbivores, pathogens) and abiotic factors can also drive natural selection on floral phenotypes (Strauss and Whittall 2006, Arista et al. 2013). Pollinator visits are usually favoured by nectar and pollen rewards, but visual signals (e.g. color, floral display, flower size) are also very important as pollinator attractants (Levin and Brack 1995, Campbell et al. 1997, Morgan and Schoen 1997, Waser and Price 1981, Jones and Reithel 2001, Parachnowitsch and Kessler 2010, Ortiz et al. 2015). In fact, the preference of pollinators for larger flowers has been shown in many studies, conferring advantages in reproductive success to individuals with larger flowers, even in gynodioecious species (Delph 1996, Williams et al. 2000, Asikainen and Mutikainen 2005, Arista and Ortiz, 2007, Van Etten and Chang 2014). Hermaphrodite flowers of gynodioecious species can

show higher attractiveness to pollinators due to both the larger flower size and pollen production, an important reward. Thus, sexual dimorphism in gynodioecious species could have critical consequences in the reproductive success of the different morphs mediated by pollinator selection (Ashman 2000). Hermaphroditic individuals should receive more pollinator visits to export the pollen, but also higher pollen loads in stigmas (Williams et al. 2000), with consequent higher fitness. In contrast, female plants with smaller flowers and without pollen as reward may be less attractive, and could suffer from pollen limitation due to lower visitation rates and the inability to self-pollinate (Williams et al. 2000, Case and Ashman 2009).

Moreover, pollinators frequently show flower constancy; that is, when visiting a plant of one morph, they are more likely to move to another of the same morph than would be expected based on morph frequencies in the population (Waser 1986). Frequency-dependent pollinator discrimination against the rare morph could also affect its maintenance in populations. Thus, females in gynodioecious species could suffer negative selection by pollinators, mainly when their number is low, affecting their reproductive success and consequent evolutionary dynamics in gynodioecious populations (Van Etten and Chang 2014). However, different pollinator species can show different preferences on floral traits, and pollinator fauna composition can change over time and population (Schemske and Bierzychudek 2001, Herrera et al. 2006). Thus, pollinator selection on females and hermaphrodites may vary according to the environmental context, a factor that is seldom considered.

The genus *Silene* L. (Caryophyllaceae) is a model system for studies in ecology and evolution (Bernasconi et al. 2009) and is commonly characterized by its diversity of sexual systems, with hermaphroditic, dioecious, gynodioecious, or gynodioecious-gynomonoecious species (Desfeux et al. 1996, Jürgens et al. 2002, Casimiro-Soriguer et

al. 2015). Monoecy has not been reported in *Silene* (Desfeux et al. 1996, Jürgens et al. 2002, Casimiro-Soriguer et al. 2015); consequently, the main evolutionary pathway to dioecy is probable via gynodioecy. In fact, gynodioecy is the second most frequent sexual system after hermaphroditism in *Silene* when including gynodioecious-gynomonoecious species within this category (Casimiro-Soriguer et al. 2016). The phylogenetic relationships within the genus are not completely resolved; consequently, evolutionary transitions between sexual systems are not completely understood. The most accepted infragenus classification is the subdivision of *Silene* into two clades, the subgenera *Silene* and *Behenantha* (Popp and Oxelman 2004, Rautenberg et al. 2010); and all the above sexual systems have been found in both subgenera at similar frequencies (Casimiro-Soriguer et al. 2015). Within the subgenus *Behenantha*, the section *Psammophilae* includes five species with low frequency of females in populations (Talavera et al. 1996, Guitián and Medrano 2000, Casimiro-Soriguer et al. 2015). Females in these species do not show a high advantage over hermaphrodites in terms of flower or fruit production (Talavera et al. 1996, Casimiro-Soriguer et al. 2013, 2015). Excepting *Silene stockenii* (Talavera et al. 1996), gender dimorphism has not been studied in this group; this subject is particularly interesting because it opens the possibility of a differential pollinator attendance of morphs.

At present there are only a few studies that have investigated the role of pollinators as agents of gender divergence in gynodioecious species (e.g. Ashman and Diefenderfer 2001, Case and Ashman 2009, Castilla et al. 2015). Given that in gynodioecious species the conditions for the maintenance of females are rather rigorous, the combination of low female frequency, low differences in fitness between morphs and negative selection on females, could originate variation in gender divergence in some populations and reversion to hermaphroditism. In the genus *Silene*, reversion from

gynodioecy to hermaphroditism has been reported at least twice (Desfeux et al. 1996). This study investigates the possibility of negative selection on female individuals by pollinators in four species of the gynodioecious *Silene* section *Psammophilae* taking into account differences among populations. To this end, we first determined if the flowers of female and hermaphrodite plants of each species showed consistent differences in size and pollen and ovule production. Pollen loads and germination on stigmas of free-pollinated female and hermaphrodite flowers were also determined. Subsequently, phenotypic selection analyses were used to ascertain if, in each species and population, female or hermaphrodite individuals were under selection by pollinators.

MATERIALS AND METHODS

Study species and study sites

The section *Psammophilae* (Talavera) Greuter of *Silene* is endemic to the Iberian Peninsula and Balearic Islands (Talavera 1979). It is composed of five annual species: *S. adscendens* Lag., *S. cambessedesii* Boiss. & Reut., *S. littorea* Brot., *S. psammitis* Link and *S. stockenii* Chater (Oxelman et al. 2013). They are all gynodioecious-gynomonoecious with female, hermaphroditic and gynomonoecious plants coexisting in the same population. Hermaphroditic and gynomonoecious plants are the most abundant, and female frequency is usually very low (Talavera et al. 1996, Guitián and Medrano 2000, Casimiro-Soriguer et al. 2013, 2015).

We studied the whole section except for *Silene stockenii*, an endangered species with only four very small populations distributed exclusively in the southwest of the Iberian Peninsula (Talavera et al. 1996, Bañares et al. 2004). For the remaining species, from two to five populations were surveyed (Figure 1). Two populations of *Silene cambessedesii* were studied (Mig and Sal); it is endemic to the Balearic Islands and

grows on sandy substrates. *Silene adscendens* inhabits in the southeastern peninsula, where three populations were sampled (Ger, Tab and Feo). *Silene psammitis* grows on dolomites or slates between 300 m and 1500 m above sea level, and three populations of the southern distribution were sampled (Ben, Oje and Jat). Finally, five populations of *S. littorea* were studied (Cas, Odi, Tra, Man and Roq), with two additional populations sampled for P:O ratio (Agu and Bre); it grows on sandy substrates along the coast of the Iberian Peninsula, from the northwest to the southeast.

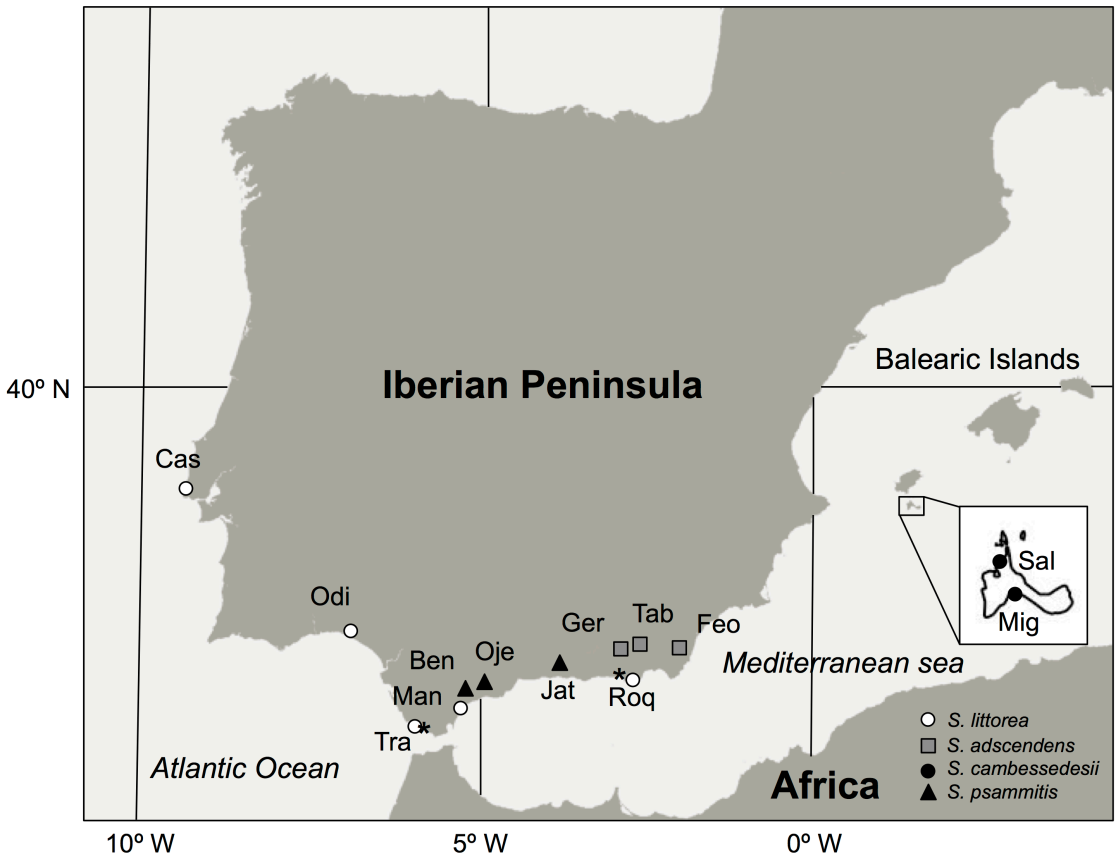

Figure 1. Populations sampled of the different species of section *Psammophilae*: three populations of *S. adscendens* (grey squares), two populations of *S. cambessedesii* (black dots), five populations of *S. littorea* (white dots) and three populations of *S. psammitis* (black triangles). Asterisks denote two populations of *S. littorea* that only were included for the P:O ratio estimates.

Flower size characterization

In order to know if female and hermaphrodite flowers differ in size, populations were surveyed in the middle of the flowering season during the spring of 2010 and 2011.

In each population, from five to eleven open-pollinated flowers of each sex were collected when stigmas were at the end of the receptive period, and preserved in FAA (ethanol: H_2O: folmaldehyde: acetic acid; 10:7:2:1).

In each flower, calyx length (CalyxL) and width (CalyxW), carpophore length (CarpophL), petal length (PetalL), limb length (LimbL) and limb width (LimbW) were measured, and ovule production counted. The three styles of each flower were preserved to determine pollen deposition and germination. From three to ten flower buds of hermaphrodite flowers were also collected to characterize the pollen:ovule ratio of each species. To this end, the number of pollen grains from one unopened anther was counted after preparation with the Avetissian micro-acetolysis method (Fægri and Iversen 1975). The number of pollen grains from one stamen was multiplied by the number of stamens on the flower to obtain the total pollen production. The number of ovules was counted under a dissecting microscope.

Pollen deposition and germination

Styles of female and hermaphrodite flowers were softened in 1N NaOH at 65ºC for 10 minutes, rinsed with distilled water for 20 minutes, and stained for 12h in decolorized aniline blue. The number of pollen grains deposited and germinated on the three stigmas of each flower was counted under a fluorescent microscope. In stigmas with more than 150 pollen grains that were saturated with pollen, this number was used as an estimate of pollen load because of the impossibility of counting the exact number.

Phenotypic selection analyses

To know if pollinators prefer hermaphrodite flowers over females due to differences in size, phenotypic selection analyses were performed in all the species using flower size (limb length) as the trait suspected to be under selection. The potential

of floral traits to evolve under pollinator-mediated selection was estimated by selection gradients and opportunities (Lande and Arnold 1983), which are useful descriptors of natural selection. Selection gradients measure the magnitude and direction of direct selection occurring on flower size, and selection opportunities measure the overall constraints on the evolution of phenotypic traits imposed by variance in fitness (Arnold and Wade 1984a, b). Selection was estimated separately for each population. Given that the greater the pollen deposition the greater ovule penetration until the maximum number of ovules is reached, we used pollen deposition, pollen deposition divided by ovule number, pollen germination and pollen germination divided by ovule number to estimate female fitness. Relative fitness was calculated for each component (the dependent variables) by dividing each absolute fitness component by its mean absolute fitness (Lande and Arnold 1983). Moreover, we standardized flower size (the independent variable) to a mean = 0 and variance = 1. Female fitness components were relativized and flower size was standardized separately for each population. Selection gradients were estimated from the standardized regression coefficients of linear regression models, in which relative fitness were regressed on the standardized trait (Lande and Arnold 1983, Conner 2001, Kingsolver et al. 2001). Lastly, selection opportunities were calculated as variances in relative fitness. All P-values were calculated with marginal (Type III) tests for significance (Arnold and Wade 1984a, b).

Data analyses

For each species, morphological differences were tested with General Linear Models with population as a random factor and the gender of the flower as a fixed factor. All variables except PetalL were transformed to assume normality (CalyxL was exponentially transformed; CalyxW, CarpophL and the number of ovules were square root transformed; and LimbL and LimbW were transformed with natural logarithm). To

determine if flower size and ovule number were correlated we performed linear regressions for each gender separately. To control for type I error due to multiple comparisons, we applied the Bonferroni correction to adjust the significance level. Theses analyses were carried out in IBM® SPSS® Statistics v.22.

Differences in the proportion of flowers pollinated and saturated with pollen (dependent variables) between flower sexes (fixed factor) were tested with a generalized linear model (GLM) assuming a binomial error distribution. To test for differences on the number of pollen grains deposited and germinated (dependent variables) between flowers sexes (fixed factor), we carried out GLMs with Poisson and binomial error distributions, respectively. The models were refitted with quasi-Poisson and quasi-binomial errors due to overdispersion of the data (Crawley 2007). For the analyses of pollen deposition all the samples were included, however for the analyses of pollen germination only those samples with at least one pollen grain on stigmas were included. All the analyses were carried out in R v.3.1.1 (R Core Team 2014).

For phenotypic selection analyses, simple linear regressions for each relativized fitness measures (response variables) with the standardized flower size (explanatory variable). These analysis were performed in IBM® SPSS® Statistics v.22.

RESULTS

Morphological measurements

Overall, *S. cambessedesii* showed the largest hermaphroditic flowers, with the largest calyx, carpophore, and petal length and widest limb, although it had the thinnest

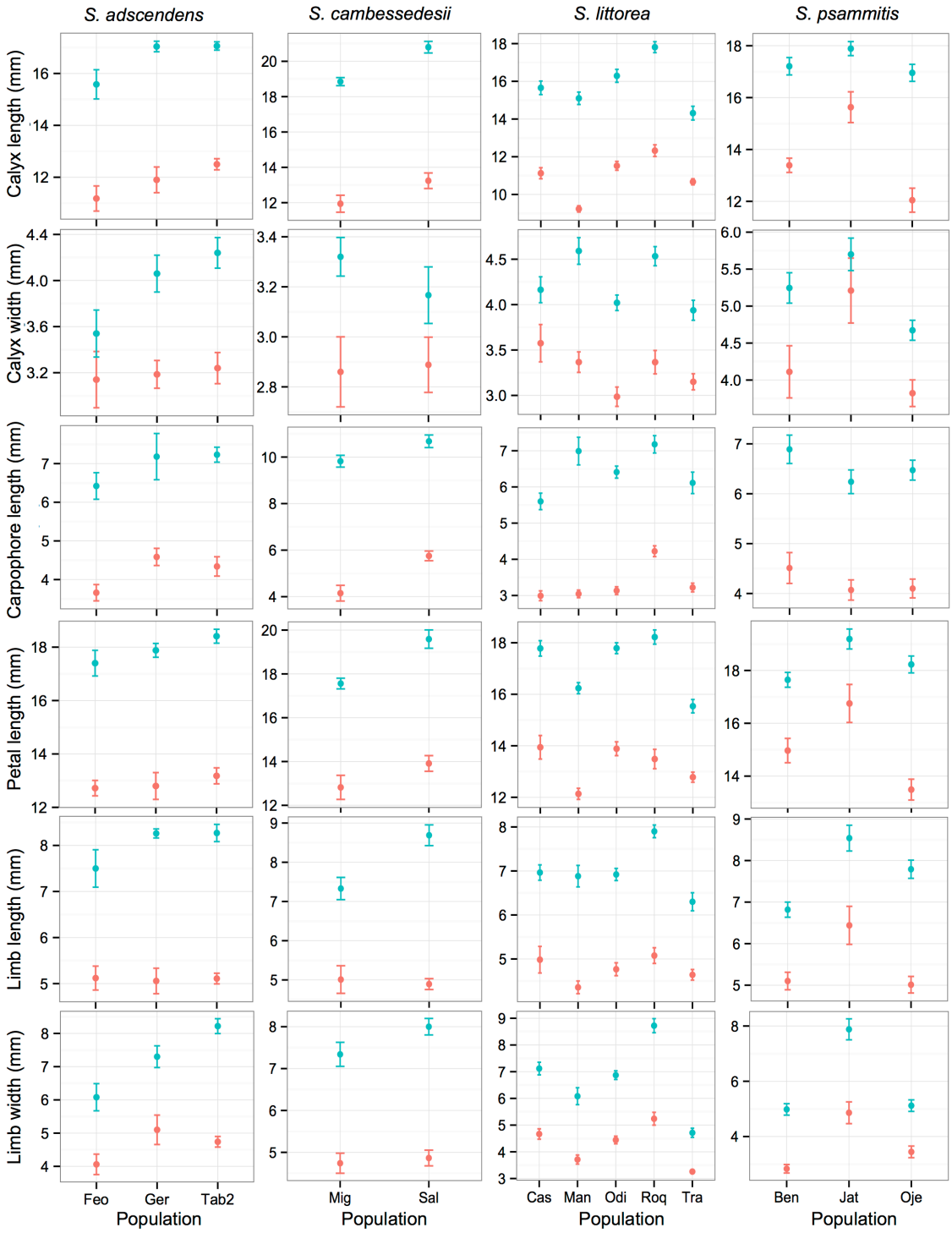

Figure 2. Morphological measurements of female (red) and hermaphroditic flowers (blue) of the species of section *Psammophilae*. Values are calculated by population and indicate mean ± 1SE of calyx length, calyx width, carpophore length, petal length, limb length and limb width. Note that Y axes have different scales.

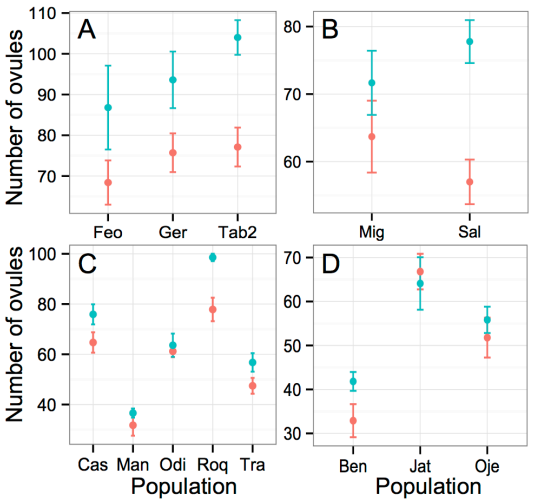

Figure 3. Mean ± 1SE of the number of ovules by population of *S. adscendens* (A), *S. cambessedesii* (B), *S. littorea* (C) and *S. psammitis* (D). Female flowers are indicated in red and hermaphroditic flowers in blue. Note that Y axes have different scales.

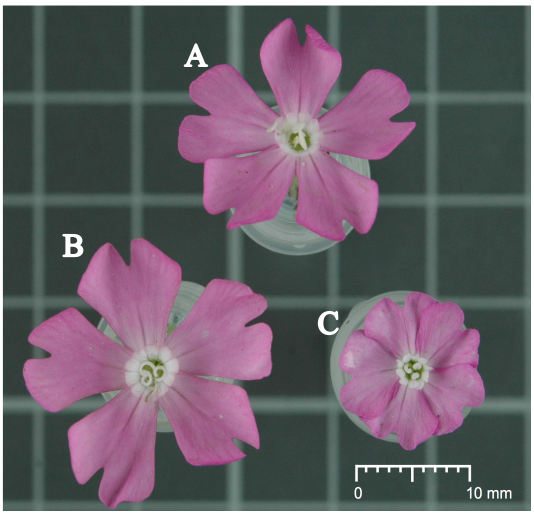

Figure 4. Hermaphroditic flower in male phase (A), hermaphroditic flower in female phase (B) and a female flower (C) of *Silene littorea*.

Table 1. Number of ovules, pollen grains and pollen-ovule ratio in hermaphroditic flower buds of species of section *Psammophilae*. Abbreviations: Pop=population, N=number of samples. In bold is highlighted the total of samples and the mean ± 1SE for all the populations of the species.

Species	Pop	N	Ovules	Pollen grains	Polen/Ovule
S. adscendens	Tab	5	91 ± 5	15592 ± 1107	172 ± 11
	Ger	5	70 ± 6	12632 ± 1481	185 ± 25
	Feo	5	61 ± 7	10362 ± 925	178 ± 23
		15	**74 ± 5**	**12863 ± 857**	**178 ± 11**
S. cambessedesii	Mig	10	65 ± 6	13872 ± 819	227 ± 17
	Sal	10	63 ± 5	12167 ± 952	203 ± 21
		20	**64 ± 4**	**13020 ± 642**	**215 ± 14**
S. littorea	Agu	5	73 ± 6	16436 ± 1504	229 ± 22
	Bre	2	45 ± 6	10900 ± 1140	249 ± 22
	Man	7	36 ± 3	9509 ± 473	277 ± 28
	Tra	3	51 ± 1	9473 ± 1021	186 ± 17
		17	**50 ± 4**	**11704 ± 910**	**243 ± 15**
S. psammitis	Jat	9	59 ± 3	13359 ± 907	231 ± 21
	Oje	10	52 ± 2	9657 ± 348	186 ± 8
	Ben	9	44 ± 4	8095 ± 491	188 ± 10
		28	**52 ± 2**	**10267 ± 528**	**201 ± 9**

calyxes. On the other hand, *S. psammitis* generally showed the biggest female flowers, with the largest calyx, petal and limb length and the widest calyxes (Figure 2). In contrast, *S. littorea* showed the shortest calyxes, carpophores, petals and limbs lengths for both, hermaphroditic and female flowers (Figure 2). *Silene adscendens* showed in general the highest number of ovules, whereas *S. littorea* and *S. psammitis* showed the lowest (Figure 3) (Table 1). Pollen production was similar between species ranging from 10267 ± 528 pollen grains (mean ± 1SE) in *S. psammitis* to 13020 ± 642 in *S. cambessedesii* (Table 1). The pollen-ovule ratio ranged from 178 ± 11 in *S. adscendens* and 243 ± 15 in *S. psammitis* (Table 1).

In general, hermaphrodite flowers were larger in size than female flowers in all the studied species (Figure 4). Significant differences were found between flower morphs ($p<0.001$) in petal length, limb length and width, calyx length and width, and carpophore length, being the hermaphroditic flowers much more larger than females in all the species (Figure 2; Table 2). Overall, significant differences between populations of each species were also found in all the traits measured (Figure 2; Table 2).

Ovule production per flower was significantly higher in hermaphroditic than female flowers in all the species except in *S. psammitis* (Figure 3). In *S. littorea* and *S. psammitis* differences between populations in ovule production were also found (Table 2; Figure 3). The linear regressions performed for each flower sex separately to analyze if larger flowers had higher number of ovules showed different patterns in each species (Figure 5). In *S. littorea* and *S. psammitis* significant positive correlation were found between flower size and ovule production in both, hermaphroditic ($R^2=0.264$, $p<0.001$, $N=47$ and $R^2=0.286$, $p=0.002$, $N=32$; respectively) and female flowers ($R^2=0.101$, $p=0.023$, $N=51$ and $R^2=0.371$, $p=0.001$, $N=28$; respectively). In *S. adscendens*, this correlation was only significant for hermaphroditic flowers ($R^2=0.370$, $p=0.004$, $N=20$),

Table 2. ANOVA of floral traits in *S. adscendens*, *S. cambessedesii*, *S. littorea* and *S. psammitis*. Significant values are highlighted in bold. Abbreviations: CalyxL= calyx length; CalyxW= calyx width; CarpophoreL= carpophore length; PetalL= petal length; LimbL= limb length; LimbW= limb width; Novules= number of ovules; Pop=Population.

Variable and source of variation	*S. adscendens*				*S. cambessedesii*				*S. littorea*				*S. psammitis*			
	df	Mean Square	*F*	*P*	df	Mean Square	*F*	*P*	df	Mean Square	*F*	*P*	df	Mean Square	*F*	*P*
CalyxL																
Sex	1	187703.99	321.40	**0.000**	1	600562.51	352.42	**0.000**	1	473924.37	550.95	**0.000**	1	182511.87	113.89	**0.000**
Pop	2	5166.04	8.85	**0.001**	1	30575.10	17.94	**0.000**	4	19263.00	22.39	**0.000**	2	22080.98	13.78	**0.000**
Error	38	584.03			42	1704.10			108	860.20			56	1602.48		
CalyxW																
Sex	1	0.49	36.62	**0.000**	1	0.12	9.68	**0.003**	1	1.72	116.55	**0.000**	1	0.60	15.57	**0.000**
Pop	2	0.04	2.70	0.080	1	0.003	0.20	0.655	4	0.09	5.75	**0.000**	2	0.37	9.62	**0.000**
Error	38	0.01			42	0.01			108	0.02			56	0.04		
CarpophL																
Sex	1	3.65	138.09	**0.000**	1	10.49	292.74	**0.000**	1	14.17	600.96	**0.000**	1	3.79	142.85	**0.000**
Pop	2	0.11	4.31	0.021	1	0.80	22.33	**0.000**	4	0.33	13.99	**0.000**	2	0.07	2.65	0.079
Error	38	0.03			42	0.04			108	0.02			56	0.03		
PetalL																
Sex	1	266.28	307.87	**0.000**	1	299.62	156.28	**0.000**	1	414.63	358.85	**0.000**	1	160.58	73.88	**0.000**
Pop	2	1.99	2.30	0.114	1	24.63	12.85	**0.001**	4	16.48	14.26	**0.000**	2	23.77	10.94	**0.000**
Error	38	0.87			42	1.92			108	1.16			56	2.17		
LimbL																
Sex	1	2.21	239.66	**0.000**	1	2.64	112.35	**0.000**	1	4.12	260.72	**0.000**	1	1.77	93.22	**0.000**
Pop	2	0.01	0.90	0.414	1	0.06	2.34	0.133	4	0.07	4.47	**0.002**	2	0.26	13.75	**0.000**
Error	38	0.01			42	0.02			108	0.02			56	0.02		
LimbW																
Sex	1	2.28	108.47	**0.000**	1	2.28	108.47	**0.000**	1	5.52	302.83	**0.000**	1	3.60	114.79	**0.000**
Pop	2	0.20	9.29	**0.001**	1	0.20	9.29	**0.001**	4	0.81	44.43	**0.000**	2	1.29	41.19	**0.000**
Error	38	0.02			42	0.02			108	0.02			56	0.03		
Novules																
Sex	1	14.54	22.98	**0.000**	1	9.59	12.55	**0.001**	1	9.20	15.63	**0.000**	1	1.27	1.59	0.212
Pop	2	1.68	2.66	0.083	1	0.08	0.11	0.745	4	33.54	56.99	**0.000**	2	19.45	24.47	**0.000**
Error	38	0.63			42	0.77			92	0.59			56	0.80		

but not for females (R^2=0.021, p=0.535, N=21). In *S. cambessedesii* flower size and the number of ovules were not significantly correlated in hermaphroditic (R^2=0.003, p=0.834, N=18) or female flowers (R^2=0.004, p=0.748, N=27; Figure 5).

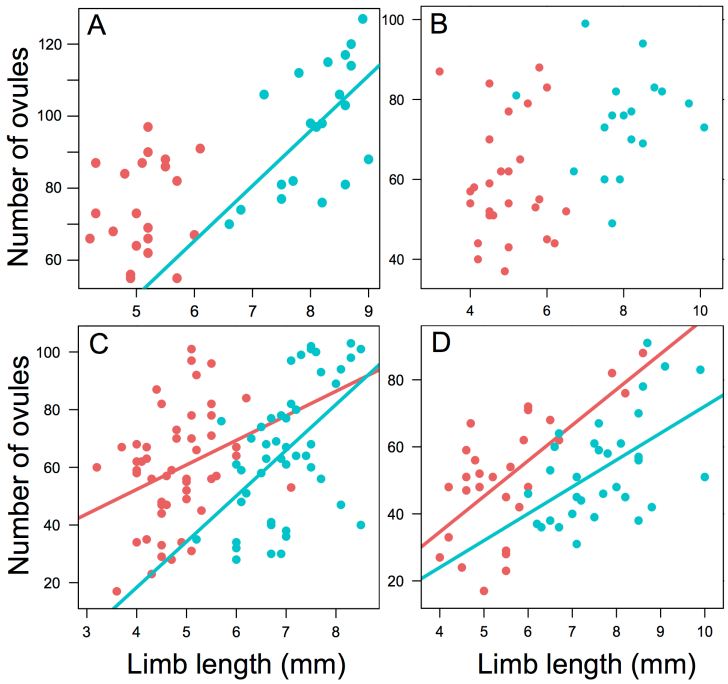

Figure 5. Regression analyses of the limb length and the number of ovules in female (red) and hermaphroditic (blue) flowers of *S. adscendens* (A), *S. cambessedesii* (B), *S littorea* (C) and *S. psammitis* (D). Note that Y axes have different scales.

Pollen deposition and germination

In general, the percentage of unpollinated flowers, i.e. flowers with zero pollen grains on stigmas, was lower in hermaphroditic than in female flowers. It ranged between 38.1% and 57.7% in female flowers and between 0% and 43.8% in hermaphroditic flowers. These differences were significant in *S. adscendens* (all hermaphroditic flowers had pollen), *S. cambessedesii* ($p<0.05$) and *S. littorea* ($p<0.001$) but not in *S. psammitis* (p=0.199)(Table 3). Similarly, the percentage of flowers with stigmas saturated with pollen was lower in female than in hermaphroditic flowers,

ranging between zero and 4.76% in female flowers and between 3.13% and 25% in hermaphroditic flowers (Table 3). However, statistically significant differences were found in *Silene cambessedesii*, *S. littorea* and marginally significant in *S. adscendens* (Table 3).

Table 3. Number of flowers analyzed, percentage of flowers unpollinated (with zero pollen grains deposited) and percentage of flowers saturated with pollen (with more than 150 pollen grains deposited in the stigmas). P-value columns show the result of generalized linear models testing for differences between flowers sexes (for further details see Materials and Methods).

Species	Number of flowers		% flowers unpollinated		P-value	% flowers saturated		P-value
	F	H	F	H		F	H	
S. adscendens	21	20	38.10	0.00	-	4.76	25.00	0.098
S. cambessedesii	28	18	40.74	10.53	*	0.00	5.26	-
S. littorea	52	47	57.69	2.13	***	1.92	23.40	**
S. psammitis	28	32	57.14	43.75	0.199	3.57	3.13	0.961

Significance values: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, - non applicable.

The generalized linear models performed to analyze the effect of sex on the number of pollen grains deposited showed significant differences ($p<0.05$) between sexes for all the species analyzed (*S. adscendens*, t=2.192, df=39, p=0.034; *S. cambessedesii*, t=3.469, df=44, p=0.001; *S. littorea*, t=4.508, df=97, p<0.001) except *S. psammitis* (t=-0.436, df=58, p=0.665). In general, hermaphroditic flowers showed higher pollen deposition than females (Figure 6). However, pollen germination in female and hermaphroditic flowers was statistically similar in most species (*S. adscendens*, t=0.058, df=31, p=0.954; *S. littorea*, t=0.844, df=66, p=0.402; *S. psammitis*, t=0.286, df=28, p=0.777) except in *S. cambessedesii* (t=-2.969, df=30, p=0.006), where the pollen showed higher germination rates in female than in hermaphroditic flowers (Figure 7).

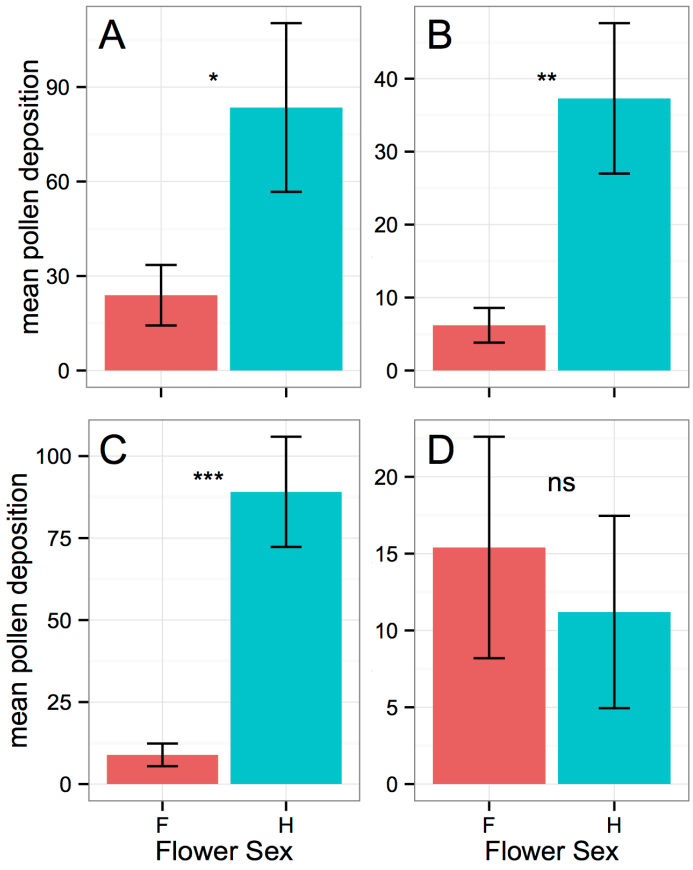

Figure 6. Mean ± 1SE of the number of pollen grains deposited on stigmas of female (red) and hermaphroditic flowers (blue) of *S. adscendens* (A), *S. cambessedesii* (B), *S littorea* (C) and *S. psammitis* (D). Asterisks denote significant differences between flower sexes in each species (*** p<0.001, ** p<0.01, * p<0.05, ns not significant).

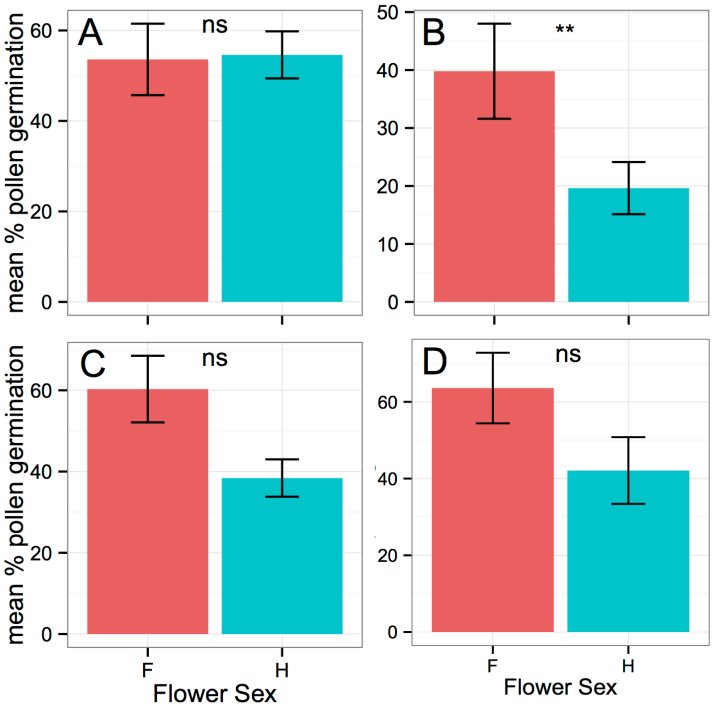

Figure 7. Mean ± 1SE of the number of pollen grains germinated on stigmas of female (red) and hermaphroditic flowers (blue) of *S. adscendens* (A), *S. cambessedesii* (B), *S littorea* (C) and *S. psammitis* (D). Asterisks denote significant differences between flower sexes in each species (*** p<0.001, ** p<0.01, * p<0.05, ns not significant).

Phenotypic selection analyses

Potentials for selection varied between populations and species. Two populations of *S. adscendens* (Ger and Feo) showed the lowest potentials or opportunities for selection for all measures of female fitness. In contrast, two populations of *S. psammitis* (Jat and Oje) showed the highest potentials for selection in three out four fitness measures (Table 4). Standardized directional selection gradients for flower size through both, absolute and relative pollen deposition, were mostly positive for all populations, indicating a trend of pollinators to select for larger flowers, except in Feo (*S. adscendens*), and Ben and Oje (*S. psammitis*) where negative selection gradients were found (Table 5). However, only four populations showed significant positive selection gradients, indicating directional selection for larger flowers in one population of *S. cambessedesii* (Mig) and in three populations of *S. littorea* (Cas, Odi and Roq). Similarly, standardized directional selection gradients for flower size through absolute and relative fitness measures of pollen germination were significant in one population of *S. cambessedesii* (Mig) and in two of *S. littorea* (Cas and Roq), indicating directional selection for larger flowers in these populations (Table 5).

Table 4. Opportunities or potentials for selection in fitness measurements (measured as the variance in the number of pollen grains deposited, number of pollen grains deposited/number of ovules, number of pollen grains germinated and number of pollen grains germinated/number of ovules) in the studied populations of species of section *Psammophilae*.

Species	Pop	N	Pollen grains deposited/number of ovules	Pollen grains deposited	N	Pollen grains germinated/number of ovules	Pollen grains germinated
S. adscendens	Tab2	19	3.160	3.627	15	1.654	1.983
	Ger	12	1.255	1.223	9	0.737	0.754
	Feo	10	0.803	0.701	9	0.673	0.656
S. cambessedesii	Mig	19	2.104	2.533	15	1.700	3.053
	Sal	26	3.446	4.048	17	1.770	3.690
S. littorea	Cas	20	2.065	1.868	13	2.337	1.916
	Man	18	1.599	1.521	12	1.576	1.532
	Odi	21	1.933	1.718	14	1.893	1.979
	Roq	22	1.266	1.282	16	2.000	2.123
	Tra	17	1.524	1.464	13	2.586	2.094
S. psammitis	Jat	20	6.985	8.468	9	2.512	3.207
	Ben	20	2.648	3.352	11	1.408	1.911
	Oje	20	6.609	6.275	10	2.686	2.470

Table 5. Standardized directional selection gradients (B' ± standard errors) for the floral size (Limb length) and fitness measurements in all populations and species of *Silene* section *Psammophilae*. Pop= population; N=samples size. Statistically significant gradients (p<0.05) are highlighted in bold. * marginally significant (p=0.05).

Species	Pop	Female frequency (%)	Pollen grains deposited/number of ovules		Pollen grains deposited	Pollen grains germinated/number of ovules		Pollen grains germinated
			N	B' ± SE	B' ± SE	N	B' ± SE	B' ± SE
S. adscendens	Tab2	11	19	0.437 ± 0.388	0.439 ± 0.415	15	0.400 ± 0.327	0.423 ± 0.354
	Ger	4	12	0.356 ± 0.331	0.426 ± 0.316	9	0.065 ± 0.324	0.163 ± 0.324
	Feo	3	10	-0.302 ± 0.302	-0.250 ± 0.287	9	-0.406 ± 0.283	-0.363 ± 0.285
S. cambessedesii	Mig	11	19	**0.529 ± 0.299**	**0.481 ± 0.338**	15	**0.575 ± 0.296**	**0.564 ± 0.305**
	Sal	9	26	0.303 ± 0.361	0.306 ± 0.391	17	-0.050 ± 0.451	-0.013 ± 0.496
S. littorea	Cas	9	20	**0.491 ± 0.424**	**0.518 ± 0.377**	13	**0.536 ± 0.389***	**0.542 ± 0.351***
	Man	18	18	0.288 ± 0.383	0.344 ± 0.357	12	-0.170 ± 0.391	-0.109 ± 0.389
	Odi	1	21	**0.532 ± 0.376**	**0.600 ± 0.315**	14	0.488 ± 0.347	0.466 ± 0.359
	Roq	13.3	22	**0.608 ± 0.225**	**0.643 ± 0.219**	16	**0.524 ± 0.322**	**0.551 ±0.325**
	Tra	5.9	17	0.149 ± 0.389	0.253 ± 0.366	13	-0.210 ± 0.474	-0.141 ± 0.432
S. psammitis	Jat	14.6	20	0.386 ± 0.575	0.345 ± 0.644	9	0.571 ± 0.492	0.501 ± 0.586
	Ben	2.2	20	-0.098 ± 0.382	-0.103 ± 0.429	11	-0.271 ± 0.381	-0.239 ± 0.447
	Oje	18	20	-0.378 ± 0.561	-0.376 ± 0.547	10	-0.609 ± 0.460	-0.607 ± 0.442

DISCUSSION

The four species of section *Psammophilae* studied have shown a marked floral dimorphism between female and hermaphrodite flowers for all traits measured. Hermaphroditic flowers were always larger than females. This pattern is consistent with the general trend in most gynodioecious species (reviewed in Eckhart 1999, Shykoff et al. 2003), and specifically in *Silene* (e.g. *S. stockenii*, *S. nutans*, *S. vulgaris*, *S. acaulis*; Shykoff et al. 2003 and references therein).

The hermaphroditic flowers produced more ovules than females, except *S. psammitis* in which ovule number was similar. This difference between sexes is infrequent in gynodioecious species, where female flowers tend to produce more ovules than hermaphrodites (Delph and Carroll 2001, Ramsey and Vaughton 2001, Sakai et al. 2013, Molano-Flores 2014), which is commonly interpreted as a female compensation through resource reallocation (e.g. Ramsey and Vaughton 2001, Sakai et al. 2013). In *S. stockenii* (the other species of section *Psammophilae* not studied here), and *S. nutans* no differences were found in ovule number between females and hermaphroditic flowers (Talavera et al. 1996, M. Dufay in Lahiani et al. 2015).

In the four species studied, both the percentage of pollinated flowers and the size of the pollen loads on stigmas were markedly lower in female than in hermaphroditic flowers. In addition, the mean size of pollen loads in pollinated female flowers was clearly insufficient to fertilize their ovules. These results indicate that female flowers of all the species suffer deficient pollination. Insufficient pollination was also found in hermaphroditic flowers of *S. psammitis*, suggesting pollen limitation in the studied populations. The greater pollen loads of hermaphroditic flowers could indicate higher pollinator attendance due to a greater attractiveness through larger size or greater pollen

and nectar rewards. Although we have not measure nectar production, pollen is an important reward for pollinators, and is only offered by hermaphrodite flowers. In other gynodioecious species it has been reported that hermaphroditic flowers show higher visitation rates than females (e.g. Asikainen and Mutikainen 2005, Alonso et al. 2007, Case and Ashman 2009, but see Arnan et al. 2014). In the *Silene* studied here, part of the pollen deposited on stigmas of hermaphroditic flowers could result from self-depositing, because there is a small overlap between the end of the male phase and the beginning of the female, as in other *Silene* species (Davis and Delph 2005, Reynolds et al. 2009, Buide et al. 2015). Thus, seeds from hermaphroditic flowers could be produced by a mixture of self- and cross-pollination, whereas seeds from female flowers are clearly from outcrossing. In support of that, the pollen:ovule ratio of these species suggests facultative autogamy (Cruden 1977, Jürgens et al. 2002). However, the level of self-pollination found in hermaphroditic flowers in previous studies seems very low: ~23% of seed set in *S. stockenii* (Talavera et al. 1996) and ~20% in *S. littorea* (Hidalgo-Triana 2010). Thus, it is very unlikely than self-depositing is responsible for the differences observed between sexes in the sizes of pollen loads. In pollinated flowers, the percentage of pollen germination was similar between sexes, except for *S. cambessedesii* where it was markedly lower in hermaphrodite flowers, probably due to a lower germination of self-pollen.

The marked differences between sexes in pollination gave rise to very great differences in relative fitness in all the species and populations studied. In consequence, selection opportunities were markedly high. Our estimates of selection gradients were relatively high compared to most published estimates of selection gradients, where most gradients were lower than 0.16 (Kingsolver et al. 2001, Hoekstra et al. 2001). In the populations studied, the absolute values of B' were generally higher than 0.16 and in

many populations they reached values greater than 0.3. The values of B' were also higher than those found for other gynodioecious species (Castilla et al. 2014). This suggests directional selection for flower size in the species and populations studied, pollinators being important selective agents in *Silene*. However, a caveat is necessary, because instead of high values for directional selection gradients, they were only statistically significant in some populations of *S. littorea* and *S. cambessedesii*. This was probably a consequence of the small sample sizes, which implies that the requirement for statistical significance is high; thus, only values higher than 0.5 were significant.

Given that, in all the species studied, hermaphrodite flowers were markedly larger than females, we could assume that selection on larger flowers indicates selection on hermaphroditic flowers. Thus, populations of *S. littorea* and *S. cambessedesii* showed positive directional selection for hermaphroditic flowers. These facts suggest that pollinators avoid visiting the females, which in consequence exhibit lower fitness. Moreover, in these species the reproductive output of the females is not much higher than that of the hermaphrodites in terms of flower number, fruit set, seed set or number of ovules (Talavera et al. 1996, Casimiro-Soriguer et al. 2015). All these facts imply important consequences for gynodioecy maintenance. Female frequency was relatively low in these populations (lower than 14%), mainly in Odi population in *S. littorea* with only 1% of females. Our results suggest that female frequency should decrease or even disappear in these populations unless an opposite force counteracts this disadvantage. In the gynodioecious *Silene nutans*, Lahiani et al. (2015) found that the relative advantage of females needed to persist, depended on the degree of pollen limitation in seed production. The hermaphroditic individuals achieved reproductive assurance only with pollen limitation, but with high pollen availability, seeds from hermaphrodites suffered from inbreeding depression, which gave the female seeds the necessary advantage to be

maintained in populations. Previous studies in *S. littorea* have found that inbreeding depression increases female frequency (Vilas and Garcia 2006), and severely limit the reintroduction success of experimental populations (Vilas et al. 2006) in the northern distribution of the species. The possibility of inbreeding depression has not been determined in our studied species, but seems a vital aspect to determine gynodioecy maintenance and evolution in this group.

CONCLUSIONS

Species of section *Psammophilae* have larger hermaphroditic flowers with a higher number of ovules and greater pollen loads than females. Moreover, larger flower sizes are strongly selected by pollinators in several populations, in detriment of female flowers, which could be suffering from pollen limitation. All these findings indicate that females, could have difficultly persisting in populations unless the avoidance of inbreeding depression confers the advantage necessary to compete with hermaphrodites.

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5

Chapter 5

The roles of female and hermaphroditic flowers in the gynodioecious-gynomonoecious *Silene littorea*: insights into the phenology of sex expression

Casimiro-Soriguer I, Buide ML, Narbona E (2013) The roles of female and hermaphroditic flowers in the gynodioecious-gynomonoecious *Silene littorea*: insights into the phenology and sex expression. *Plant Biology* 15:941–947.

ABSTRACT

Some gynodioecious species have intermediate individuals that bear both female and hermaphroditic flowers. This phenomenon is known as a gynodioecious-gynomonoecious sexual system. Gender expression in such species has received little attention in the past, and the phenologies of male and female functions have also yet to be explored. In this study, we examined variations in gender patterns, their effects on female reproductive success and sex expression in depth throughout the flowering period in two populations. The studied populations of *Silene littorea* contained mostly gynomonoecious plants and the number of pure females was very low. The gynomonoecious plants showed high variability in the total proportion of female flowers. In addition, the proportion of female flowers in each plant varied widely across the flowering season. Although there was a trend towards maleness, our measures of functional gender suggested that most plants transmit their genes via both pollen and ovules. Fruit set and seed set were not significantly different among populations; in contrast flower production significantly varied between the two populations - and among plants - with consequent variation in total seed production. Conversely, gender and sex expression were similar in both populations. Plants with higher phenotypic femaleness did not have higher fruit set, seed set or total female fecundity. The mating environment fluctuated little across the flowering period, but fluctuations were higher in the population with low flower production. We therefore conclude that the high proportion of gynomonoecious individuals in our studied populations of *S. littorea* may be advantageous for the species, providing the benefits of both hermaphroditic and female flowers.

KEYWORDS

Female reproductive success, functional gender, mating environment, phenology of sex expression, standardised phenotypic gender.

INTRODUCTION

Gynodioecy is a sexual system in which plants bearing hermaphroditic flowers coexist within a population with plants that produce only female flowers (Webb 1999). Gynodioecy is considered a possible intermediate stage in the pathway from hermaphroditism to dioecy (Charlesworth and Charlesworth 1978), and possible scenarios for the evolution from hermaphroditism to gynodioecy and from gynodioecy to dioecy have been investigated (Charlesworth 1999). The theoretical predictions about the evolution of gynodioecy depend on the genetic mechanism involved in the sex determination of flowers: male sterility can be determined by nuclear genes (Schultz 2002), or as the result of mitochondrial genes that cause male sterility interacting with nuclear restorers of male fertility (nuclear-cytoplasmic gynodioecy; Bailey et al. 2003). Purely nuclear inheritance in plants is rare; in most gynodioecious species analysed sex determination implicates both cytoplasmic and nuclear genes (Bailey and Delph 2007). Models for the maintenance of nuclear-cytoplasmic polymorphism consider both the female fitness component (female advantage) and the cost of restoration (negative fitness of hermaphrodites with an excess of restorers; Gouyon et al. 1991, Bailey et al. 2003). Three main proximal causes of female advantage have been proposed: the reallocation of resources, sex differences in interactions with herbivores and reduced inbreeding depression in females (Dufay and Billard 2012).

In species with nuclear-cytoplasmic inheritance of gynodioecy, incomplete restoration at several nuclear loci causes partial male sterile individuals (e.g. plants with hermaphroditic and female flowers; Koelewijn and Van Damme 1995). Although frequently neglected, these "gynomonoecious" individuals are common in many gynodioecious species (e.g. Horovitz and Galil 1972, Collin and Shykoff 2003), especially in the genus *Silene* (Talavera et al. 1996, Charlesworth and Laporte 1998, Maurice 1999, Lafuma and Maurice 2006; Dufay et al.

2010). These species are considered to have a gynodioecious-gynomonoecious sexual system (GGSS; Desfeux et al. 1996, Dufay et al. 2010).

Sex expression in gynodioecious-gynomonoecious populations should be measured as a continuum rather than merely as a categorical trait. Lloyd (1980a,b) proposed two measures: phenotypic gender, which quantifies an individual plant's expenditure in pollen and seeds; and functional gender, which also considers the production of gametes and seeds by other individuals in the population. Knowledge of functional gender is important for understanding processes related to the evolution or maintenance of the gynodioecious sexual system (Verdú et al. 2004, Delph and Wolf 2005), because in the case of male biased functional gender in hermaphrodites, selection should increase allocation towards male function, leading to the evolution of dioecy (Maurice et al. 1993). In addition, functional gender makes it possible to include gynomonoecious plants as a continuum between functional male and functional female plants in species with GGSS (Lloyd and Bawa 1984). In this sense, the inclusion of gynomonoecious individuals in the analysis of functional gender is fundamental in clarifying their role in the evolution of this sexual system.

Estimates of functional gender include the mating environment (i.e. the relative number of ovules available to pollen that can potentially fertilise them; Lloyd 1980b, Brunet and Charlesworth 1995), which can vary across the flowering season. In particular, species with sexually dimorphic flowers or with dichogamy are expected to experience high variations in the mating environment (Thomson and Barrett 1981, Brunet and Charlesworth 1995). In gynodioecious species, the mating environment depends both on the number of ovules available from hermaphroditic flowers in the female phase and on the number of ovules available from female flowers. Estimating the mating environment in species with GGSS is more complex because it can also be affected by variation in female and hermaphroditic flower production in gynomonoecious plants. Even more complexity can

occur if the species exhibits dichogamy. For example, in species with protandry, the mating environment can fluctuate widely during the flowering season, being male biased in the beginning of the season, and the opposite being true in species with protogyny (Wells and Lloyd 1991, Brunet and Charlesworth 1995; but see Thomson and Barrett 1981, Narbona et al. 2011). To date, the flowering phenologies of the different morphs or the phenologies of male and female functions in species with GGSS have received little attention (Maurice 1999, Widén and Widén 1999); furthermore no analysis of dichogamy has been incorporated in these studies.

Silene is considered a model genus in evolutionary biology (Bernasconi et al. 2009). The highly variable sexual systems in this genus are particularly interesting; either gynodioecy or hermaphroditism are considered the ancestral condition, and dioecy has evolved at least twice (Desfeux et al. 1996, Marais et al. 2011). *Silene littorea* has nuclear-cytoplasmic inheritance of gynodioecy (Vilas and García 2006), in common with other congeners like *S. vulgaris* and *S. nutans* (Taylor et al. 2001, Garraud et al. 2011). Previous studies have shown that the relative frequencies of the three sexual phenotypes (females, gynomonoecious and hermaphroditic plants) are highly variable among populations (Guitián and Medrano 2000). In this study, we examined the GGSS of *S. littorea* in a phenological context to assess whether standardized phenotypic gender (see Material and Methods for definition) and the mating environment of the flowers vary across the blooming period. Because of the low number of females found in the populations, we will quantify female advantage through a correlation between femaleness (measured as standardized phenotypic gender) and fecundity. The study was performed using two natural populations with contrasting habitats. Our specific objectives were to: (i) compare phenotypic and functional gender among plants and populations, (ii) examine the relationship between gender

expression and female reproductive success, and (iii) assess flower production, phenotypic gender and mating environment throughout the flowering period.

MATERIALS AND METHODS

Study system

Silene littorea ssp. *littorea* Brot. (Caryophyllaceae) is an annual plant that grows in coastal dune ecosystems from the northwestern to the southeastern Iberian Peninsula (Talavera 1979). The inflorescence is a monochasium, and the number of flowers per plant is highly variable (ranging from three to 338 in this study). *Silene littorea* is self-compatible (Vilas et al. 2006). This entomophilous species produces protandrous hermaphroditic flowers with almost no overlap between sexual phases; the durations of the male and female phases are 3-4 and 3-5 days, respectively.

In the spring of 2010, two populations from the south of Spain were intensively examined from the beginning to the end of flowering. The first, "Faro de Trafalgar", hereafter Trafalgar (36°10'58.3" N 6°1'58.8" W, Cádiz, Spain), is located in a coastal sand dune ecosystem with scattered shrubs such as *Juniperus phoenicea* ssp. *turbinata* and *Pistacia lentiscus*. The second population, "Pinares de la Breña", hereafter Breña (36°11'26.1"N 5°56'35.3"W), is also located on a sandy substrate, but is situated in a *Pinus pinea* forest.

Flowering phenology and production and female reproductive success

Before the onset of flowering, 60 plants were marked in each of the Trafalgar and Breña populations (54 and 58 plants survived, respectively). Plants were examined weekly from the beginning (19 February) to the end (4 June) of the blooming period. On each census day, the number of female and hermaphroditic flowers in anthesis was counted on each plant. We also annotated the sexual phase (male or female) of the hermaphroditic flowers. Each new flower was marked with a coloured tag to avoid re-counting the same flower.

Female reproductive success was measured as fruit set (total fruits/total number of flowers produced by each marked plant) and seed set (seeds per fruit/ovules per flower). In female and hermaphroditic plants, seed set was estimated from four non-dehiscent fruits per plant when they matured, at the end of the flowering season. Similarly, in gynomonoecious plants, seed set was calculated from two fruits from female flowers and two fruits from hermaphroditic flowers. Because this species is annual, the total number of seeds produced is a measure of the lifetime female fecundity. Thus, we estimated total female reproductive success per plant as the mean of seeds/fruit x total number of fruits produced.

Estimation of gender and temporal variation of the mating environment

We used three measures of the plant gender: proportion of female flowers, temporal standardised phenotypic gender, and functional gender. The standardised phenotypic gender is a modification of phenotypic gender proposed by Lloyd (1980b), which measures a plant's expenditures in pollen and ovules relative to the average ratio of expenditure across the population (Lloyd and Bawa 1984). Wells and Lloyd (1991) and Sato (2002) have proposed a modification of standardised phenotypic gender to account for temporal variation in sex expression across the flowering season in species with dichogamy. We adapted the formula of Wells and Lloyd (1991) to determine the temporal standardised phenotypic gender (hereafter temporal phenotypic gender) of *S. littorea,* which has both female and hermaphroditic flowers and in which male and female sexual phases of hermaphroditic flowers can be differentiated due to their marked protandry. We calculated the temporal phenotypic femaleness for each plant and each census day (T_{it}) and in the whole flowering period (T_i) as:

$$T_{it} = f_{it} / \{f_{it} + (m_{it} * E_t)\}$$

$$T_i = \Sigma_t f_{it} / \{\Sigma_t f_{it} + \Sigma_t (m_{it} * E_t)\}$$

$$E_t = \Sigma_i f_{it} / \Sigma_i m_{it}$$

where f_{it} and m_{it} are the numbers of female phase flowers (hermaphroditic flowers in female phase and female flowers) and male phase flowers (hermaphroditic flowers in male phase), accordingly, of an individual *i* each sampling day *t*. E_t is the ratio of female phase to male phase flowers each sampling day *t* for all *i* plants of the population. Following Sato (2002) and Ehlers and Thompson (2004), the equivalence factor E_t is taken as an estimate of the mating environment, defined as the ratio of ovules available to pollen that can potentially fertilise them at time *t* across the entire population (Brunet and Charlesworth 1995). T_i can vary between 0 and 1 (a plant produces only pollen or ovules at time *t*, respectively).

Functional gender measures the proportion of a plant's fitness that is transmitted through ovules or pollen, (Lloyd 1980b). In this study, we estimated functional femaleness (G_i) as:

$$G_i = d_i / \{d_i + (l_i * \bar{E})\}$$

where d_i is the total number of fruits produced per flowers with a gynoecium (females and hermaphroditic flowers) and l_i is the total numbers of flowers with an androecium (hermaphroditic flowers) of an individual *i* during the flowering period (Delph and Lloyd 1991). We incorporated the mean mating environment ($\bar{E} = \Sigma_t E_t / n$; where *n* is number of sampling days), which accounts for the mean proportion of female phase flowers to male phase flowers in the population (Brunet and Charlesworth 1995, Sato 2002). Realistic estimates of functional gender must incorporate additional information about male or female success (e.g. paternity analysis and posterior progeny quality; Elle and Meagher 2000, Verdú et al. 2004), or must be based on certain assumptions. Following Lloyd (1980b) and Lloyd and Bawa (1984), equal success of pollen grains and ovules and absence of self-fertilisation must be assumed for calculation of functional gender using flower and fruit estimates. In *S. littorea* we can only meet the first requirements because we cannot rule out self-fertilisation (E. Narbona and N. Hidalgo, unpublished results).

Data analysis

We used generalized linear models to determine the effect of T_i and population on female reproductive success (fruit set, seed set and number of seeds per plant), assuming binomial errors (logit link function) for the variables fruit set and seed set, and Poisson errors (log link function) for the number of seeds per plant. We used T_i instead of G_i to avoid autocorrelation for the number of fruits. Models were fitted using quasi-binomial and quasi-Poisson errors due to the over-dispersion of the data (Crawley 2007). We tested the association between femaleness (T_i) and total production of flowers with Spearman correlations because data normalization after transformation was not achieved. The same analysis was carried out to test the association between male and female sexual functions across the flowering season. All analyses were carried out in R 2.12.0 (R Development Core Team 2010).

RESULTS

Gender expression and flower production

In both populations most individuals were gynomonoecious. In the Trafalgar population, 3.7% of plants were females, 7.4% were hermaphrodites, and 88.9% were gynomonoecious. In the Breña population, no plants were exclusively female, 19.0% were hermaphrodites, and 81.0% were gynomonoecious. In gynomonoecious individuals, we observed a high variability in the total proportion of female flowers produced per plant, which ranged from 1.3% to 81.1% (mean ± s.e. = 13.6% ± 2.3) in the Trafalgar population, and from 4.2% to 66.7% (21.1% ± 2.1) in the Breña population.

Temporal phenotypic femaleness at the plant level is shown in Figure 1A. At Trafalgar, T_i ranged from 0.23 to 1; at Breña, T_i ranged from 0.24 to 1. In both populations, plants showed T_i values that continuously varied within the range with insignificant gaps, with

the exception of a few plants that had values representing a tendency toward complete femaleness. Only one completely female plant (T_i = 1) was found in Trafalgar, and two in Breña.

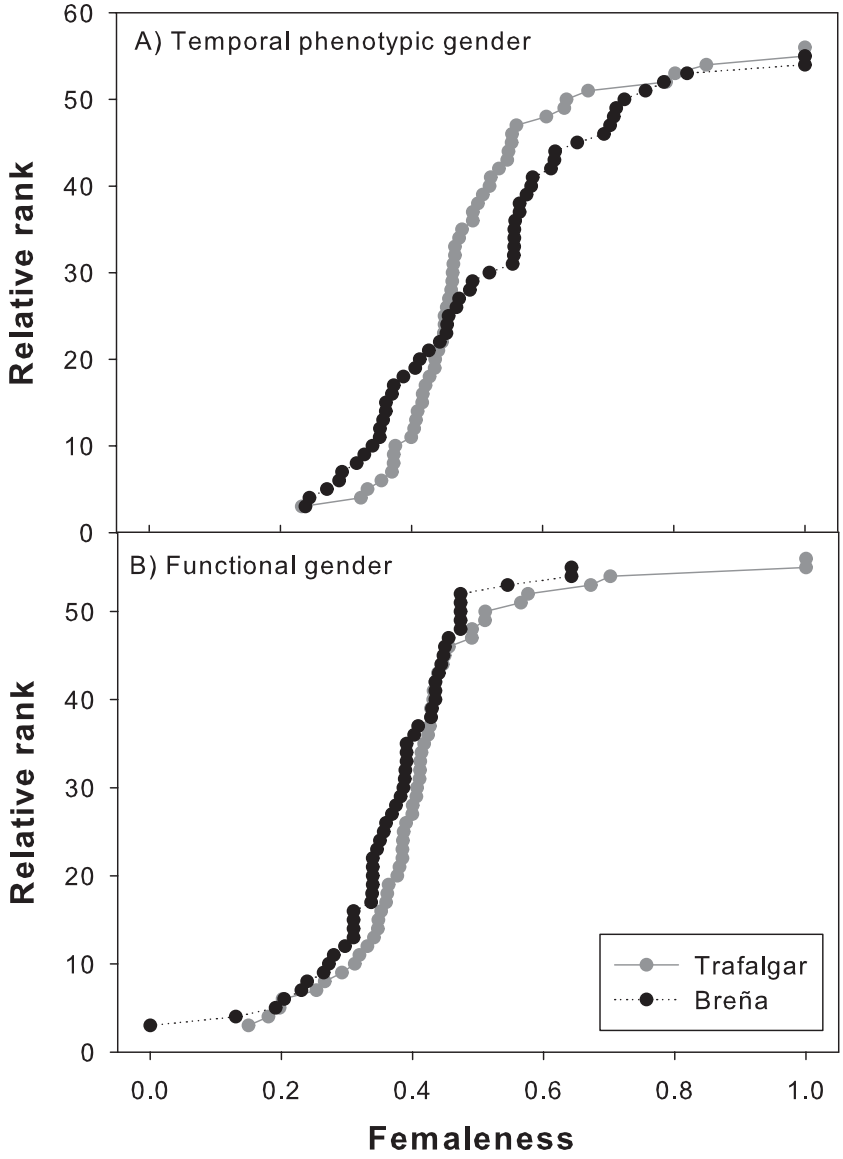

Figure 1. Temporal phenotypic gender (A) and functional gender (B) in Trafalgar (grey circles) and Breña (black circles) populations. Plants were ordered by the relative value.

Functional femaleness showed very similar values in both populations, with the exception of plants with extreme values (Fig. 1B). At Trafalgar, G_i values ranged from 0.15 to 1, while at Breña, G_i ranged from 0 to 0.64. Most plants had values that continuously varied between 0.3 and 0.5 in both populations. The Trafalgar population contained only two

functionally female plants (G_i = 1) and no functionally male plants (G_i = 0), while the Breña population contained not completely female plants but one functionally male plant.

The total flower production of plants in the Trafalgar population was six times higher than that of the Breña population (97.4 ± 9.1 and 17.7 ± 1.9 flowers, respectively). Within populations, the flower production per plant was highly variable, ranging from five to 338 in Trafalgar and from three to 69 in Breña. Total flower production was not significantly correlated with T_i either at Trafalgar (R^2 = 0.009, P = 0.48) or in the Breña population (R^2 = -0.036, P = 0.17).

Effects of gender expression on female reproductive success

The relative measures of female reproductive success (fruit and seed set) were highly similar in both populations, and no significant differences were detected (Table 1). At Trafalgar, the mean fruit and seed set were 55.9 ± 1.7 (n = 54) and 67.3 ± 2.1 (n = 29), respectively. At Breña, the corresponding values were 60.8 ± 2.4 (n = 58) and 65.2 ± 1.9 (n = 19). T_i did not affect fruit set or seed set (Table 1).

Table 1. Results of the analysis of deviance for the effect of femaleness and population on the female reproductive success (fruit set, seed set and estimated number of seeds per plant).

Response variable		df	Deviance	Residual df	Residual Deviance	F	P
Fruit set	T_i	1	2.157	105	342.73	0.684	0.410
	Pop.	1	3.050	104	339.68	0.968	0.327
Seed set	T_i	1	0.137	46	167.12	0.038	0.846
	Pop.	1	0.016	45	167.10	0.004	0.948
Seeds per plant	T_i	1	631	46	134348	0.313	0.391
	Pop.	1	56817	45	77532	28.26	<0.001

The absolute measure of female fecundity, the estimated number of seeds per plant, was statistically different between populations (Table 1), with a mean of 3741 ± 625 (n = 29) seeds/plant at Trafalgar and, 600 ± 125 (n = 19) at Breña. In addition, plants within each population varied considerably in absolute female fecundity, particularly at Trafalgar, where the estimated number of seeds per plant ranged from 536 to 14208. At Breña, these values were lower, ranging from 188 to 2331. These differences between populations can be attributed to the variability in flower production per plant. As before, the relationship between T_i and the number of seeds per plant was not significant (Table 1).

Phenology of sex expression

The proportion of female flowers produced by each individual plant varied enormously across the flowering period [see **Supporting Information**: Figure S1, Table S1, Table S2].

The mean temporal phenotypic femaleness of the plants (Fig. 2A) in Trafalgar fluctuated from 0.37 to 0.63, with most values around 0.5; it should be noted that from April 25 to the end of flowering the values showed a continuously rising trend. At Breña, the mean temporal phenotypic gender for each census day ranged from 0.33 to 0.69; although in the second half of the flowering period, the values at Breña showed higher variation between consecutive censuses than those in Trafalgar.

The variation in the mating environment during the flowering season is shown in Figure 2B. The ratio oscillated between 0.49 and 1.75 (mean 0.71) at Trafalgar, and between 0.33 and 2.43 (mean 0.92) at Breña. The pattern of variation in the mating environment was similar in both populations, despite the wider fluctuations at Breña.

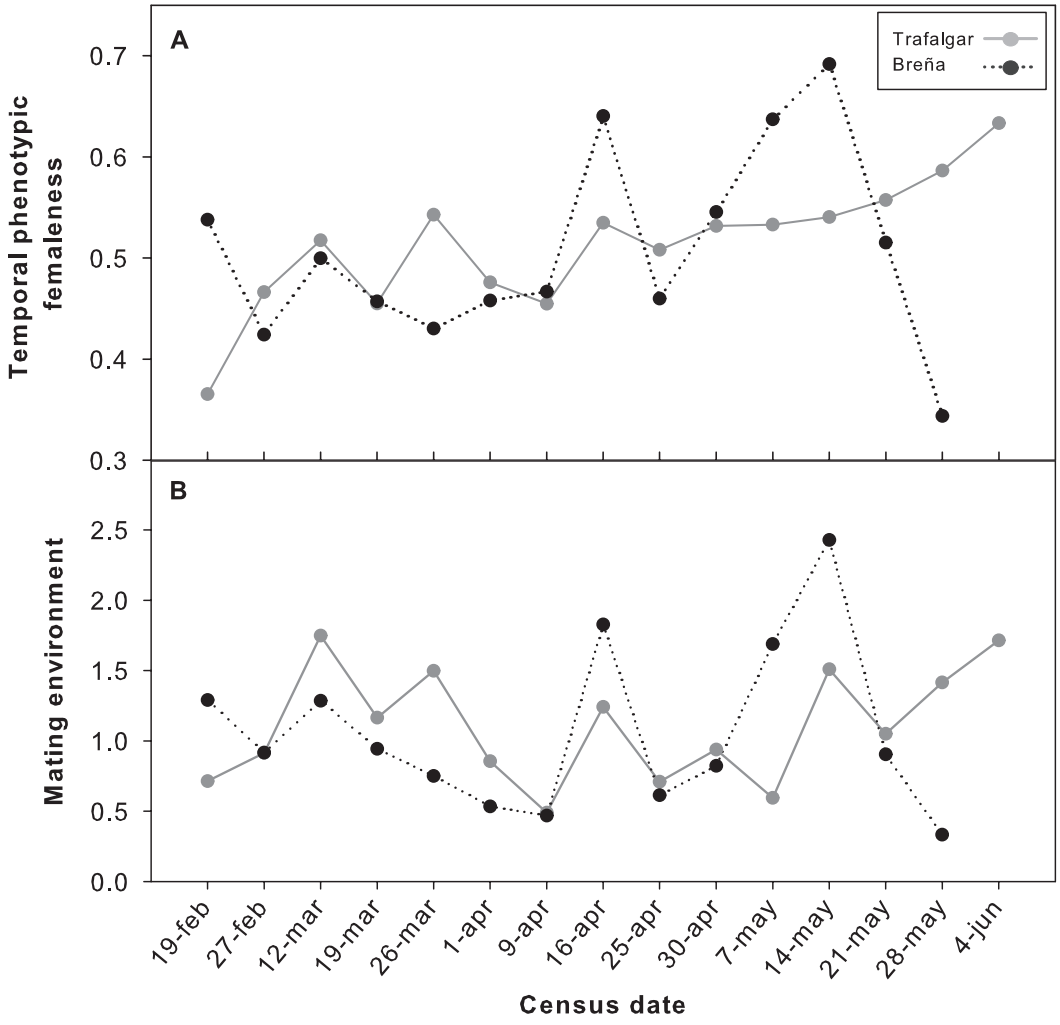

Figure 2. Temporal phenotypic gender (A), and mating environment (B) for each census day throughout the flowering period in Trafalgar (grey circles, solid line) and Breña (black circles, dashed line) populations. Mating environment is the ratio of female phase (hermaphroditic flowers in female phase and female flower) to male phase flowers (hermaphroditic flowers in male phase).

The number of hermaphroditic flowers in the male phase varied across the flowering season, showing three peaks at Trafalgar and four at Breña (Fig. 3A and 3B). The number of hermaphroditic flowers in the female phase varied similarly across the flowering season (Fig. 3A and 3B). The number of female flowers was lower in both populations, but was not uniform over the time studied. In the Trafalgar population, the number of hermaphroditic flowers in male phase was positively correlated with both the number of flowers with female function (hermaphroditic flowers in female phase plus female flowers) ($R^2 = 0.70$, P = 0.004),

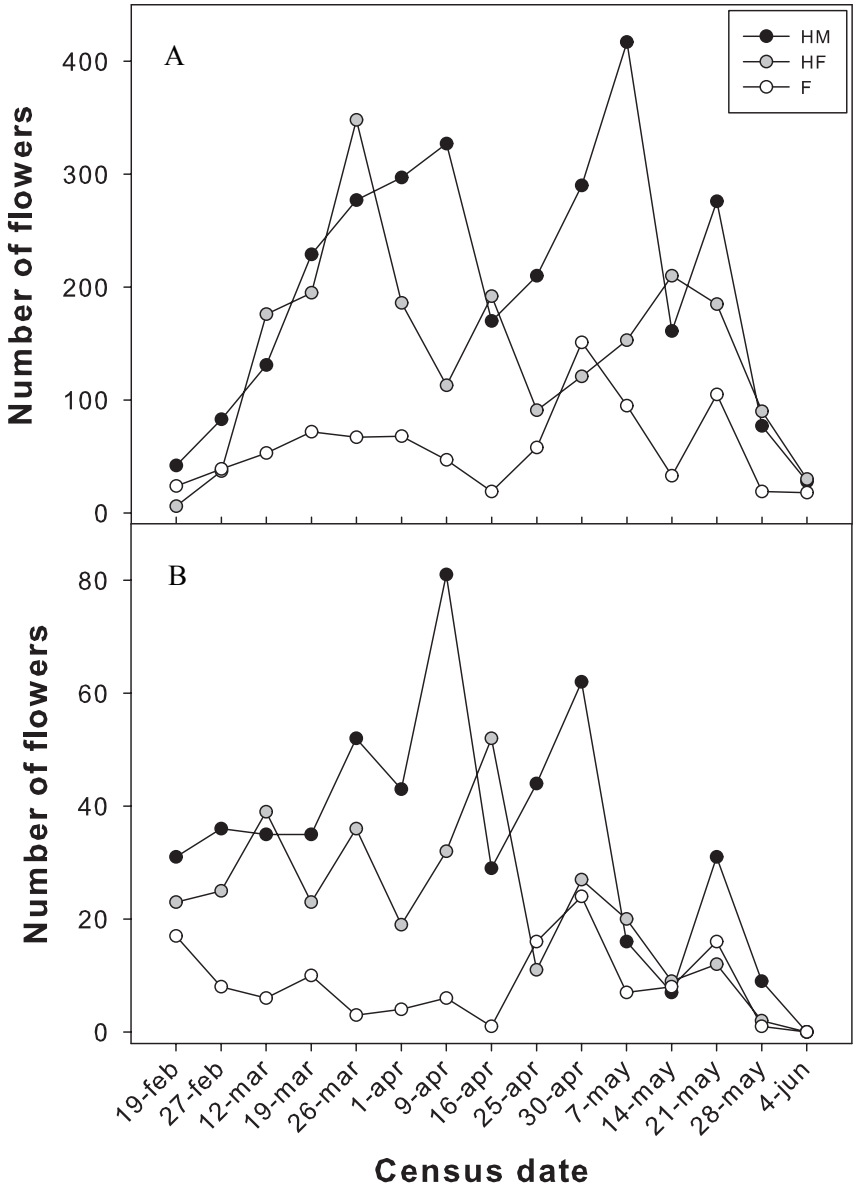

Figure 3. Number of total female and hermaphroditic flowers in male and female phase during the flowering season in Trafalgar (A) and Breña (B) populations. Black circles are hermaphroditic flowers in male phase (HM). Grey circles are hermaphroditic flowers in female phase (HF). White circles are female flowers (F).

and the number of female flowers (R^2 = 0.71, P = 0.003). Similarly, at Breña, the number of hermaphroditic flowers in male phase was marginally correlated with the number of hermaphroditic in female phase plus female flowers (R^2 = 0.55, P = 0.041) but not with the numbers of hermaphroditic flowers in female phase and female flowers taken separately (P = 0.13 and 0.38, respectively).

DISCUSSION

One of the main results of this study is that in the gynomonoecious-gynodioecious populations of *Silene littorea,* most plants were gynomonoecious (between 81-89%). However, in other GGSS species, hermaphroditic plants are generally found in similar or higher proportions compared to gynomonoecious plants (Koelewijn and Van Damme 1996, Maurice 1999, Dufay et al. 2010). A high proportion of gynomonoecious plants has been detected in other populations of *S. littorea* in the northern Iberian Peninsula (mean 51%, Guitián and Medrano 2000) and in other closely related species, such as *S. stockenii* (40%, Talavera et al. 1996). By analysing flower sex expression throughout the flowering period, we have observed that apparently female or hermaphrodite plants may become gynomonoecious when tracked across the complete flowering period, because they produced a few hermaphroditic or female flowers, respectively, in only one or a few censuses.

In species with GGSS, some hypotheses have been proposed to explain the evolutionary significance of gynomonoecious individuals. Under certain circumstances, female flowers of gynomonoecious plants can partially avoid inbreeding depression by favouring outcrossing rates, whereas the hermaphroditic flowers may provide pollen to offset pollen limitation (Cheptou et al. 2001). Due to incomplete protandry, hermaphroditic flowers of *S. littorea* can self-fertilize, although in a low proportion, when styles grow and touch the last anthers (E. Narbona and N. Hidalgo, unpublished results). Autonomous selfing, which assures reproduction, can be selected in areas with uneven pollinator visitation (Davis and Delph 2005), such as the coastal dune ecosystems where this plant grows. Thus, the presence of female flowers of gynomonoecious plants of *S. littorea* could increase the outcrossing rates; whereas hermaphroditic flowers can help to maintain the reproductive assurance when pollinator activity is scarce, which may explain the high proportion of gynomonoecious individuals in our analysed populations. Therefore, as Davis and Delph (2005) found in *S.*

noctiflora, gynomonoecy in *S. littorea* can be a system that allows for mixed mating, combining the advantages of both selfing and outcrossing in the same individual.

We have found that pure females are extremely rare in Trafalgar population, and absent altogether in Breña. In addition, we did not find any increase in either fruit set, or seed set, or total number of seeds per plant with increasing levels of femaleness. Thus, female plants cannot be said to have an apparent advantage in female fertility, which may explain the low observed frequencies of female plants in the populations analysed of *S. littorea*. Nevertheless, other aspects of female fecundity, such as seed mass, germination rate or seedling survivorship, should be analysed. In a recent review, Dufay and Billard (2012) state that female advantage is predominant in the gynodioecious species analysed - although notable exceptions to this prevalent pattern are *Daphne laureola* (Alonso and Herrera 2001), and *S. stockenii* (Talavera et al. 1996).

This study shows that, in addition to being the most frequent morph, gynomonoecious individuals vary widely in their proportion of female flowers, both among plants and within each plant across the flowering period. When we consider the behaviour of other plants in the population (i.e. T_i), gynomonoecious plants show values in a continuum between 0.2 and 1, indicating that there are no gender specialized individuals in the population. The G_i values show a similar pattern, but most plants have values between 0.3 and 0.5, which indicates that these plants function as hermaphrodites, but transmit a slightly higher proportion of their genes *via* pollen than *via* ovules.

In *S. littorea,* the pattern of sex expression phenology is complicated not only by protandry but also by the variation in the proportion of female flowers produced by individual gynomonoecious plants and by the multiple peaks of flowering across the flowering period. In species with dichogamy at the flower or plant level, the differential phenologies of male and female functions usually result in considerable variation in the mating environment (Barrett

1984, Méndez and Díaz 2001, Narbona et al. 2011), sometimes with sharp oscillations or with changes of more than 10-fold during the season (Thomson and Barrett 1981, Wells and Lloyd 1991). In *S. littorea*, the mating environment of each population continuously fluctuated within a certain range during the flowering period and with no strong variation from the beginning to the end of flowering, which suggests that there is pollen available to fertilize ovules at any time of the flowering period. However, the mating environment in Trafalgar showed a three- to four-fold variation, whereas Breña displayed more than seven-fold. The population with plants producing a significantly higher number of flowers, Trafalgar, showed lower fluctuations in the mating environment. Therefore, the contrasting population at Breña had lower values of absolute female reproductive success, directly related to the number of flowers, but also a less balanced mating environment. These differences between the populations may be due to the fact that the Breña plants grow under dense pine or scrub cover, and as a result suffer resource depletion due to shading (Stanton et al. 2000).

In conclusion, *S. littorea* populations exhibit a GGSS in which the most frequent morph is gynomonoecious individuals with labile production of hermaphroditic and female flowers. Female plants are rare in the populations studied, suggesting that the species are nearer to the gynomonoecious than to the gynodioecious sexual system. However, this GGSS would allow an increase in female plants if population condition change (Emery and McCauley 2002, Bailey and McCauley 2005, Vilas and García 2006). Measures of gender variation indicate that only a few plants act exclusively as females or males. Instead, plants exhibit a continuum in phenotypic gender with most values near 0.5, and are slightly skewed to maleness in measures of functional gender. The mating environment fluctuated throughout the flowering period within narrow limits, but with higher fluctuations in the population with low flower production. We suggest that the GGSS in *S. littorea* allows for mixed mating, combining the advantages of both selfing and outcrossing. In addition, the phenology of sex

expression should be taken into account to explain the possible adaptive advantage of GGSS, which is not uncommon in *Silene* spp. (Desfeux et al. 1996).

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SUPPORTING INFORMATION

Table S1: Proportion of female flowers of a plant in each census date in Trafalgar population.

	Census date														
Plant	19 feb	27 feb	12 mar	19 mar	26 mar	1 apr	9 apr	16 apr	25 apr	30 apr	7 may	14 may	21 may	28 may	4 jun
1	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.67	0.71	0.25	0.00	0.00		
2	0.00					0.00	0.00	0.00							
4		1.00	0.33	0.00	0.00	0.11	0.00	0.00	0.25	0.14	0.45	0.00	0.00	0.00	0.40
5	0.00	0.00	0.25	0.00	0.13	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.14	0.00	0.00
6		1.00	0.00	0.00	0.00	0.17	0.25	0.00	0.00	0.00	0.20	0.22			
7		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.25	0.33	0.17	
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.25	0.00		0.50	
9	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
10	0.67	0.60	0.13	0.00	0.00	0.00	0.00	0.00	0.30	0.33	0.17	0.00	0.14	1.00	
11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00		0.00	0.25	0.00		
12	0.33	0.00	0.00	0.00	0.08	0.00	0.00	0.08	0.21	0.21	0.06	0.14		0.00	
13	0.00	0.25	0.14	0.17	0.04		0.01	0.00	0.04		0.13	0.04	0.06	0.06	0.00
14		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.18	0.09	0.00	0.50		
15	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.14	0.18	0.50	0.17	1.00	0.50	
16			0.00	0.00	0.00	0.00	0.00		0.50	0.00	0.20	0.57	0.00	0.00	0.00
17		0.67	0.00	0.11	0.11	0.10	0.08	0.00	0.00	0.29	0.08	0.08	0.32	0.00	0.15
18		0.00	0.33	0.29	0.38	0.11	0.50	0.00	0.44	0.90	0.33	0.00		1.00	
19	1.00	0.17	0.00	0.10	0.00	0.00				0.67	0.20	0.14	0.45	0.00	0.00
20	1.00		0.00	0.00	0.20	0.00	0.00	1.00	0.00	0.22	0.00	0.00	0.07	0.00	
21				0.00	0.20	0.00		0.00	1.00	0.38	0.00	0.00	0.14		
22	0.00	0.25	0.08	0.17	0.00	0.25	0.67	0.00		0.67	0.15	0.00	0.23		
23	0.00	0.14	0.00	0.08	0.00	0.60	1.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	
24	0.50	0.86	0.60	0.67	0.71	0.73	0.86	0.00	0.82	0.80	0.36	0.60	0.83	1.00	
25	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00		1.00
28			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.15	0.00	
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.55	0.25	0.00	0.47	0.13	0.00
30	1.00	1.00	0.29	1.00	0.20	0.11	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.14	0.23	0.00	0.03	0.50	
32		0.00	0.10	0.00	0.00	0.06	0.00		0.50	0.00	0.10	0.00	0.00		
35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.17	0.00	0.00	0.00	
36	1.00	0.00	0.00	0.14	0.00	0.00	0.00	0.60	0.00	0.33	0.13	0.00	0.00	0.67	
37	0.00	0.00	0.50	0.67	0.00	0.00	1.00			1.00	1.00	0.00	0.14		
38	1.00	0.00	0.00	0.00	0.08	0.17	0.00	0.00		0.75	0.00	0.00	0.17	0.00	
39	0.00	1.00	0.14	0.20	0.04	0.00	0.00	0.36	0.17	0.26	0.07	0.14	1.00		
40					0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
41			0.00	0.00	0.00	0.00	0.00			1.00	0.20	0.00	0.00		
42	0.00	0.00	0.00	0.00	0.00	0.00				0.00	0.00	0.00	0.00		
43		0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.08	0.13	0.00	0.20	0.00	0.00	1.00
44	0.33	0.07	0.00	0.14	0.06	0.00	0.05	0.00	0.00	0.36	0.13	0.13	0.05	0.00	
45	0.00	0.00	0.11	0.00	0.22	0.13	0.05	0.00	0.05	0.09	0.18	0.00	0.14	0.00	
46	0.00	0.00	0.00	0.00	0.33	0.50	0.00	0.00	0.00	0.33	0.17	0.00	0.33	0.00	
47			0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00
48	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.20	0.00				
49			0.00		0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.33		
50		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	
51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.30	0.00	0.00	0.50	0.00	0.25
52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.05	0.00	0.00
53		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.18	0.33	0.00	0.50	0.00	

54	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	
55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.05	0.00	0.00	0.00	1.00
56	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.67
57	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.40		
58	0.67	0.00	0.00	0.00	0.00	0.08	0.09	0.00	0.05	0.00	0.00	0.00	0.50	0.00	
59	1.00	1.00	1.00	1.00	1.00	1.00	1.00			1.00	1.00	1.00	1.00		
60	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00		1.00		

Table S2: Proportion of female flowers of a plant in each census date in Breña population.

	Census date												
Plant	19 feb	27 feb	12 mar	19 mar	26 mar	1 apr	9 apr	16 apr	25 apr	30 apr	7 may	14 may	21 may
1	0.00	0.00	0.00	0.00			0.00						
2	0.00	0.00	0.00	0.33			0.00	0.00		1.00	1.00		
3				1.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	1.00
4	1.00	0.00	0.00	0.00		1.00							0.00
5	0.00	0.00			0.00	0.00		0.00	0.00	0.00	0.00	0.00	1.00
6	0.00		0.00	0.50	0.00	0.00					1.00		0.00
7	0.00	0.00			0.00	0.00				1.00			
8	0.00			0.00							1.00	1.00	0.00
9	0.00	0.00				0.00							
10	1.00		1.00	0.00			0.00						
11	0.00	0.00	0.00		0.00	0.00			1.00	1.00			
12	0.00	0.50		0.00					0.00				
13	1.00	0.00		0.00		1.00		0.00	0.00	0.00			
14	0.00	0.00							0.00				0.00
15	0.00	0.00			0.00	0.00	0.00		0.00	0.00	0.00		
16							0.00	0.00	0.00	0.00			0.00
17	0.00	0.00		1.00	0.00					0.33	0.00		
18	1.00	0.00	0.00		0.00					1.00	1.00	0.00	
19	0.00	0.00	0.00	0.00			0.00		0.00	0.00	0.00		0.75
20	1.00	0.50	1.00		0.00	0.00	0.00	0.00	0.00	0.20	0.00		0.00
21	0.00	0.00	0.00	0.00	0.00	0.13	0.08	0.00	0.00	0.14	0.00		
22	1.00	1.00									0.00		
23	1.00		0.00	0.00									
24	1.00	0.00	0.00		0.00	0.50	0.00	0.00	0.00	0.00		1.00	0.00
25	1.00	0.00	0.00		1.00		0.50	0.00	1.00	0.50	0.25	0.67	0.71
26				0.00		0.00	0.00	0.00	0.00			0.00	
27	0.00	0.00	0.00			0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00
28	0.00	0.00	0.25	0.33	0.00	0.00	0.08	0.00	0.50	0.00			
29	0.00	0.00	0.00	0.00	0.00	0.00							
30	0.00	0.00	0.00	0.50	0.00	0.00	0.00		0.33	0.00	0.00		
31		0.00	0.00	0.00	0.00	0.00				1.00	0.00	1.00	0.00
32	0.00		0.00	0.00	0.00	0.00	0.00	0.00	1.00				
33		0.00	0.00	0.00	0.00		0.00	0.00					
34	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.14	0.00			
35		1.00	0.67	1.00	0.00	0.00	0.00		0.50	0.50			
36	1.00	0.50		0.00	0.00		0.50	0.00	0.00			0.00	0.50
37	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.33	0.00	0.00	0.00
38	0.00	0.00	0.00	0.00	0.00		1.00	0.00	0.00			0.00	
39	0.00	0.00								0.00	0.00		0.00
40	0.00					0.00	0.00		0.00	1.00	0.00		
41	0.00		0.00	0.50					1.00				
42	0.00							0.00		0.00			1.00
43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.50		0.00	0.00
44	0.00	0.00	0.00	0.00			0.00			0.00	0.00	0.00	0.00
45	0.00		0.00		0.00				0.00	1.00	0.00		
46	0.00	0.00						1.00					
47	0.00	0.50	0.00	0.00	0.00				0.50	0.00	0.00		
49	0.00	0.00		0.00	0.00	0.00	0.00	0.00	1.00	0.00			
50	0.00			0.00							1.00		1.00
51	1.00	0.00	0.33										

52	0.00	0.00	0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00
54	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.33	0.00	0.00	
55	0.00	0.00			0.00		0.00		0.00	0.00			0.00
56	0.00	0.50	0.00		0.00	0.00	0.00	0.00	0.75	0.20	0.00		0.00
57	1.00		0.00	0.40	0.20	0.00	0.00	0.00	0.33	0.50	0.00	0.00	1.00
58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.50			
59			0.00		0.00	0.00		0.00		0.00		0.00	
60	1.00	0.00	0.00	0.00	0.00		0.00		0.50				

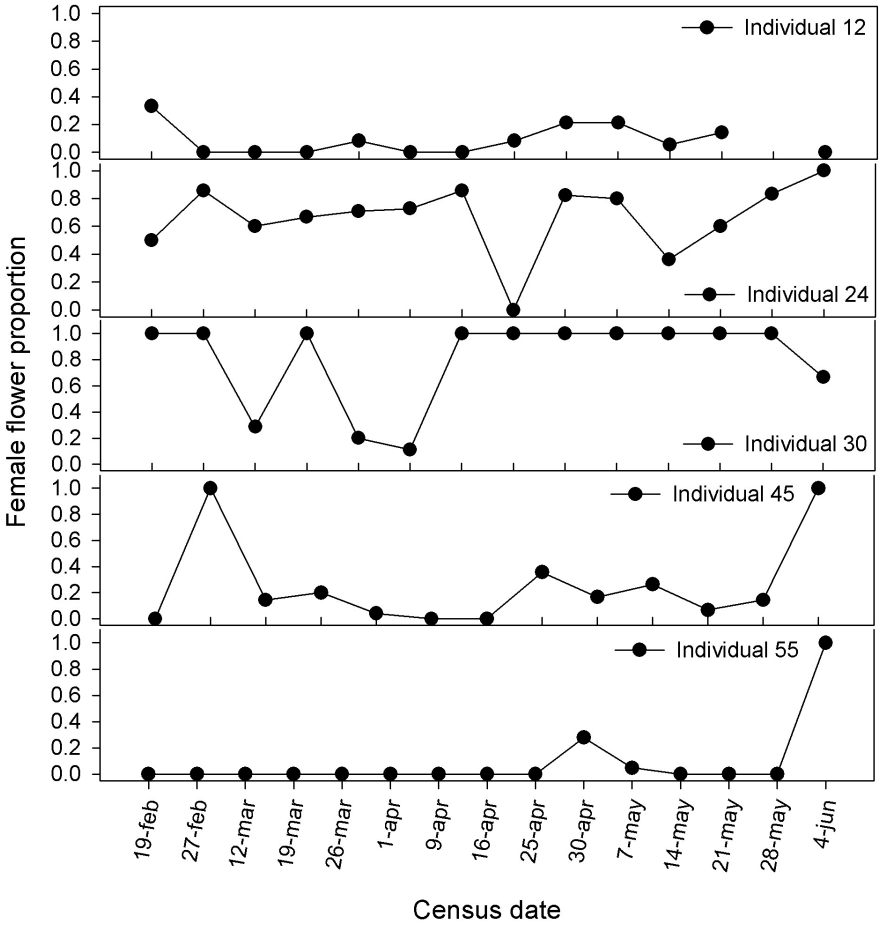

Figure S1: Proportion of female flowers of five PMS plants of Trafalgar population during the flowering period showing labile sex expression of plants in *Silene littorea*. Plants were selected because they represent different trends of variation.

6

Chapter 6

Transcriptome and biochemical analysis of a flower color polymorphism in *Silene littorea* (Caryophyllaceae)

Casimiro-Soriguer I, Narbona E, Buide ML, del Valle JC, Whittall JB. Transcriptome and biochemical analysis of a flower color polymorphism in *Silene littorea* (Caryophyllaceae) (submitted to *BMC Genomics*).

ABSTRACT

Background: Flower color polymorphisms are widely used as model traits from genetics to ecology, yet determining the biochemical and molecular basis can be challenging. Anthocyanin-based flower color variations can be caused by at least 12 structural and three regulatory genes in the anthocyanin biosynthetic pathway. We use mRNA-Seq to simultaneously sequence and estimate expression of all of these candidate genes in nine samples of *Silene littorea* representing the three distinct petal color morphs (dark pink, light pink and white) across three developmental stages. We then compare the mRNA-Seq results with the biochemistry of the petal. We report SNP differences, expression profiles and biochemical differences among the three petal color morphs of *S. littorea*.

Results: We identified 29 putative paralogues for the 15 candidate genes in the anthocyanin biosynthetic pathway. We assembled complete coding sequences for 16 structural loci and complete coding sequences for nine of ten regulatory loci. Among these 29 putative paralogues, we identified 622 SNPs, yet only nine synonymous SNPs in *Ans* had allele frequencies that differentiated pigmented petals (dark pink and light pink) from white petals. We also found one locus with strong differential expression (more than 42x) in *F3h* between pigmented morphs and white samples. *C4h2*, *F3'h* and *Myb1a* also presented differential expression between different color morphs, but to a lesser degree. Biochemical profiles revealed cyanidin as the primary anthocyanin and five flavonoid intermediates. Concentrations of three of the flavonoid intermediates were significantly different between pigmented and white petals (rutin, quercetin and isovitexin).

Conclusions: RNA-Seq successfully sequenced and simultaneously estimated expression differences of all 15 candidate genes in the anthocyanin biosynthetic pathway was performed in the non-model species *S. littorea*. We identified several synonymous SNPs surrounding an intron in *Ans* that requires further investigation. We also found several loci with significantly lower expression in white petals, although the most dramatic expression difference is in *F3h*. The biochemistry results for rutin, quercetin, luteolin and apigenin are consistent with a blockage at F3H or above. Lastly, a tentative anthocyanin biosynthetic pathway for petals of *S. littorea* is proposed.

KEYWORDS

Silene littorea, Anthocyanin biosynthetic pathway, Flavonoid biochemistry, mRNA-Seq, HPLC, Flower color polymorphism, Transcriptome, Anthocyanin synthase, Flavanone-3-hydroxylase.

BACKGROUND

Flower color has played a pivotal role in our current understanding of biology since Mendel's discovery of the inheritance of flower color in *Pisum sativum* (Mendel 1866, Ellis et al. 2011). Since then, flower color has contributed to our understanding of such diverse aspects of biology such as gene regulation (Napoli et al. 1990), pleiotropy (Streisfeld and Rausher 2011), population genetics (Wright 1943, Schemske and Bierzychudek 2001), speciation (Bradshaw et al. 1995, Hopkins and Rausher 2011) and ecology (Irwin and Strauss 2005; Eckhart et al. 2006; Strauss and Whittall 2006). Although many discoveries utilize model species with complete genomes, numerous evolutionary and ecological questions regarding flower color variation reside in non-model species. These investigations would benefit from an efficient method for sequencing and detecting expression of all flower color related genes in non-model species.

The most common floral pigments are the anthocyanins (Miller et al. 2011; Campanella et al. 2014) which are produced by the anthocyanin biosynthetic pathway (ABP). Floral anthocyanins are now considered a metamodel because of the conserved nature of the biosynthetic pathway across most angiosperms (Kopp 2009). Changes in the color of anthocyanins (e.g., shifts from blue to red flowers) and the loss of floral anthocyanins (producing white flowers) can now be traced from ecological interactions in the field to the biochemical and molecular basis for these changes (Tanaka et al. 2008, Davies 2009, Hopkins and Rausher 2011). These changes can result from mutations in core structural genes or regulatory loci (Sobel and Streisfeld 2013).

The ABP is composed of seven core enzymes and several side-branching enzymes and appears largely conserved across angiosperms (Holton and Cornish

1995, Grotewold 2006). Blockages in the pathway can be divided into early and late halves depending on whether flavonols are produced. Because of the stress-responsive nature of the flavonols, early blockages are predicted to have more dramatic physiological and potentially ecological consequences compared to late blockages that still produce flavonols (Whittall et al. 2006). The consequences of blocking the ABP can be ameliorated by recruiting tissue-specific regulators and decreasing expression solely in the petal (Streisfeld and Rausher 2011, Wessinger and Rausher 2012, Sobel and Streisfeld 2013). The ABP is regulated by a complex composed by three interacting transcription factors: the R2R3-MYB, the basic helix-loop-helix (bHLH) and the WD40-repeat (WDR) (Hichri et al. 2011). The MYBs confer the majority of the tissue specificity (Stracke et al. 2001, Dubos et al. 2010). Collectively, the core ABP, side-branches within the ABP, genes leading into the ABP and regulatory genes provides a relatively large target for a diversity of mutations that could block the ABP (Wessinger and Rausher 2012). For flower color polymorphic plants, locating the blockage and predicting the physiological and ecological consequences require a thorough characterization of the ABP at the biochemical and molecular scales (e.g. Lou et al. 2014, Nishihara et al. 2014). Disentangling the type of mutations that cause the loss of anthocyanins in flower color polymorphic species is a complicated task because it requires the sequencing of all genes acting in the ABP and their regulators.

RNA-Seq is a fast and efficient approach to sequence and examine the expression of all ABP-related genes, even when a reference genome is not available as in most non-model species (Li et al. 2012, Lulin et al. 2012, Xu et al. 2013, Butler et al. 2014). For flower color polymorphisms, petal mRNA must be examined across a range of developmental stages, especially the earliest stage when the flower color

polymorphism is manifested (Whittall et al. 2006, Dick et al. 2011, Butler et al. 2014). Large, complex genomes often exhibit multiple paralogues, sometimes expressed in the same tissue (e.g. Martins et al. 2013, Yuan et al. 2014). Differentiating paralogues and getting paralogue-specific expression levels can be a complicated step in the mRNA-Seq bioinformatics pipeline. Once a candidate gene has been identified with either sequence or expression differences that correlate with flower color, subsequent biochemical analysis of the petal can be used to test the flavonoid composition and confirm the location of the blockage in the ABP. High-Performance Liquid Chromatography (HPLC) coupled with mass spectrometry has been extensively used to identify and quantify the flavonoid composition in many ornamental and wild plants (Fossen and Andersen 2006, Qiao et al. 2011). For instance, high concentrations of anthocyanins in black cultivars of *Dahlia*, were related with elevated expression of the ABP genes and low concentrations of flavones (Thill et al. 2012).

The genus *Silene* (Caryophyllaceae) is a model for studies of evolutionary ecology (Bernasconi et al. 2009), yet no one has examined the molecular and biochemical basis for flower color polymorphisms in any of the species (yet see the proposed pathway in Kaamsteeg et al. 1979). Although the Caryophyllales are largely characterized by the production of betalain pigments in place of anthocyanins, flower color variation in *Silene* is still caused by the omnipresent anthocyanins (Brockington 2011). Herein, we focus on a discrete flower color polymorphism in the Iberian Peninsula endemic, *S. littorea* (Talavera 1979). After surveying 17 populations across the species range, we found most populations were dark pink (Figure 1A). In two northern populations, there were three distinct color categories: white, light pink, and

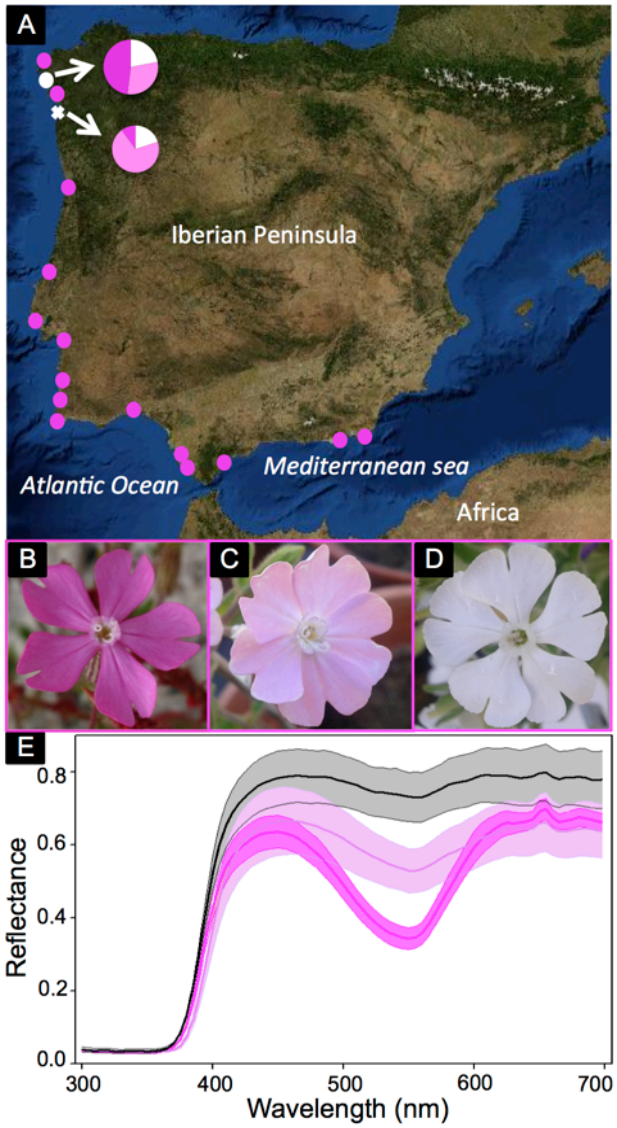

Figure 1. The map in (A) shows the populations sampled of *S. littorea* from the northwest coast to the southeast coast of the Iberian Peninsula. The single polymorphic population (Playa de Barra) sampled for RNA-Seq and biochemical analysis is indicated with a white cross. The two polymorphic populations are indicated with white symbols and the proportions of white-, light pink-, and dark pink-flowered individuals in each population are illustrated with pie diagrams (the number of individuals counted in each category is also indicated). Pink circles indicate populations fixed for dark pink petal color. The three distinct color morphs are illustrated in B-D: dark pink (B), light pink (C) and white (D). The average UV-VIS spectral reflectance of the upper surface of the petals of six dark pink samples (dark pink line), six light pink samples (light pink line) and three white samples (black line) are indicated in (E) with standard errors (shadow area).

dark pink (Figure 1B-D). The differences between the three petal color morphs was confirmed with UV-Vis spectra of the petals (Figure 1E).

Here, we examine the petal transcriptome and biochemistry of the flower color polymorphism in *S. littorea* using RNA-Seq complemented with HPLC flavonoid profiling. Transcriptome analysis is used to sequence and estimate expression of fifteen ABP-related genes. The sequences of these candidates are examined for color differentiating synonymous and non-synonymous SNPs. Simultaneously, we estimate expression differences between color morphs to determine if downregulation of any ABP-related genes correlate with white petals. We complement our RNA-Seq results with an investigation of the petal biochemistry of the three color morphs by identifying the primary anthocyanin pigment and flavonoid intermediates. We then

compare their relative abundances among the three color morphs to help target the blockage in the ABP leading to white petals. The biochemical results are interpreted in light of the SNP and expression findings from the transcriptome analysis.

MATERIALS AND METHODS

Plant species

Silene littorea (Caryophyllaceae) has an anthocyanin petal polymorphism with three distinct categories – dark pink (P), light pink (L) and white (W) (Figure 1B-D). It belongs to the section *Psammophilae* (Oxelman et al. 2013), that is composed of five annual, primarily pink flowered taxa. The species grows primarily in coastal sand dunes of Spain and Portugal (Figure 1A; Talavera 1979). It has a gynodioecious-gynomonoecious sexual system and produces a highly variable number of flowers per plant (Casimiro-Soriguer et al. 2013), yet the flower color is constant among flowers within a plant (unpublished observations). There is no correlation between flower color and sexuality (unpublished observations), but white individuals are much more common in the northwestern portion of the species range compared to the southeast (Figure 1A).

Sampling and RNA extraction

Flower petals were sampled from a polymorphic population near the northwestern range limit (Playa de Barra, Spain; 42º 15’ 35’’N, 8º 50’ 25’’W) (Figure 1A). Flower petals were sampled from W, L and P flowers in three developmental stages: bud, opening, and anthesis (Additional file 1). All five petals from the same flower were collected and immediately preserved in RNA*later* (Ambion, Inc., Austin, Texas) and stored at -20 ºC until the RNA could be extracted. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen,Valencia, CA).

Concentration and purity of RNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and agarose gels were run to verify RNA integrity. The nine samples with the highest concentration of RNA for each of the three color morphs and developmental stages were selected: bud white, opening white, anthesis white, bud light, opening light, anthesis light, bud pink, opening pink and anthesis pink.

Library preparation and sequencing

RNA-Seq libraries were prepared and sequenced at the Epigenome Center of the University of Southern California following the manufacturers protocol (Illumina, San Diego, CA). The nine libraries were barcoded (6bp), pooled in equimolar concentrations and loaded on a single lane of the Illumina Hi-Seq 2000 system. Sequencing consisted of 50 cycles of single-end sequencing-by-synthesis reactions. Fortuitously, our libraries were sequenced twice. After a preliminary analysis indicating similar results were attained from each individual dataset, we merged the two datasets. In the combined dataset, there was an average of 37.9 million reads per sample (range 36.3 – 40.0 million).

***De novo* assembly of ABP-related loci**

Since there are no closely related genome resources for *Silene*, we conducted *de novo* assembly of the *S. littorea* transcriptome following a similar pipeline developed by Butler et al. (2014), using the *de novo* assemblers VELVET v.1.2.07 (Zerbino and Birney 2008) and OASES v.0.2.08 (Schulz et al. 2012). We used the FASTQ files generated by the Illumina Hi-Seq 2000 platform with a range of *k*-mers (23-39) to compare the assemblies. Assembled contigs were identified by BLAST+ against the *Arabidopsis thaliana* RefSeq database from TAIR (v. 10) (Swarbreck et al. 2008), limiting the results to e-values $<10^{-10}$. For each of the nine samples, we

extracted all contigs from the candidates for each gene of the ABP and these contigs were aligned in BioEdit (Hall 1999). For several genes, distinct isoforms of the same gene were unalignable, yet blasted to the same ABP-related locus in the TAIR database. These isoforms were treated separately to avoid grouping paralogous sequences and we then produced a single consensus sequence for each isoform (hereafter "locus") with ambiguities representing all the variable sites among the nine samples.

To simplify our sequence comparisons and expression analyses, we limited ourselves to 15 ABP-related genes. These include seven core ABP structural genes: chalcone synthase (*Chs*), chalcone isomerase (*Chi*), flavanone-3-hydroxylase (*F3h*), dihydroflavonol 4-reductase (*Dfr*), anthocyanidin synthase (*Ans*), glycosyl transferase (*Uf3gt*), acyltransferase (*At*); three genes immediately upstream of the ABP: phenylalanil ammonia-lyase (*Pal*), cinnamic acid 4-hydroxylase (*C4h*), coumarate CoA ligase (*4Cl*); two side-branching genes: flavonol synthase (*Fls*), flavonoid 3'hydroxylase (*F3'h*), and three transcriptional regulators: basic helix loop helix (*Bhlh*), WD40 Repeats (*Wd40*) and R2R3-MYB domains (*Mybs*).

Sequence comparisons of ABP-related loci

To examine sequence correlations with flower color, we identified single nucleotide polymorphisms (SNPs). We started by mapping the microreads back onto the *de novo* consensus sequences using Mosaik (Lee et al. 2014). We followed the author's recommendations for the parameter settings: two allowed mismatches and a hash length of 15. We then used Picard v.1.94 (http://broadinstitute.github.io/picard/) to identify PCR duplicate reads – an artifact of the library preparation methodology. The Genome Analysis Toolkit v.2.6-4 (GATK, McKenna et al. 2010) was used to (1) re-align the reads around potential indels, (2) remove the PCR duplicates identified in

Picard and finally (3) identify SNPs in each of the nine samples across the 29 ABP-related loci (DePristo et al. 2011). We calculated the allele frequencies for each SNP for each color type using the genotype field (GT) in the GATK output file (we surveyed 3 individuals per color morph = 6 alleles per color morph). We also calculated the mean likelihood of genotype assignment (0/0, 0/1 or 1/1) for each color type (parameter PL in the GATK output file).

Expression analysis

To extract the number of reads mapped for each gene from the bam file produced by Mosaik we used Artemis (Rutherford et al. 2000). For differential expression analysis we used the DESeq package (Anders and Huber 2010) in R (R Core Team 2013). This package requires the normalization of the raw counts. After normalization, only those loci that pass the quantile 33 threshold are further analyzed for differential expression. This filtration is neccesary to avoid spourious stimates of fold-change values due to very low expression values. Then, negative binomial tests were applied to find the statistically differential expressed loci ($p < 0.05$). Although we have analyzed the three developmental stages separately, we will specially focus on the bud stage, because the color is already present in the bud (Additional file 1), and the genes will be primarily acting in this developmental stage.

Phylogenetic analysis of R2R3 *Myb*s

To help infer which *S. littorea* petal *Myb*s may be involved in anthocyanin biosynthesis, we compared the six distinct *Mybs* identified in *S. littorea* to known regulators of the ABP from several model species (*Antirrhinum majus*, *Arabidopsis thaliana*, *Gerbera hybrida*, *Ipomoea nil*, *Lycopersicon esculentum*, *Malus domestica*, *Petunia hybrida* and *Vitis vinifera* – accession numbers can be found in Additional file 2). Bayesian phylogenetic analysis of the aligned nucleotids of the R2R3 binding

domain (303 bp) was conducted in MrBayes v.2.0.8 (Huelsenbeck and Ronquist 2001). We applied the GTR+I+G model of sequence evolution for two separate runs, each consisting of four independent chains with 5,000,000 generations of sampling after 1,000,000 generations of burnin. Trees were sampled every 50000 generation. Convergence and mixing were assessed in Tracer.

HPLC analysis of flavonoids

Flavonoid's identification was studied in three dark pink samples. After that, we proceeded to quantify each specific compound in three white, six light pink and six dark pink samples. Flavonoids were extracted from four petals of a flower, in anthesis stage, that were preserved in 1 mL of $CH_3OH:H_20$ (9:1, v:v) containing 1% HCl, and kept on ice and in the dark. Samples were homogenized using 5 x 3mm glass beads in a Mixer Mill MM 200 (Retsch, Haan, Germany) with a frequency of 30 oscillations/s. They were beaten until the sample was completely homogenized (minimum of 60 s). The supernatant was removed after 10 min centrifugation (13,000 rpm) and stored at -20ºC until separated by HPLC.

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of a hybrid triple-quadruple linear ion trap (QqQLIT) mass spectrometer equipped with an electrospray ion source. HPLC analyses were performed on a 150 X 2.0 mm Phenomenex Luna 3u C18(2) 100A reversed-phase column with a particle size of three μm. The flow rate was 0.2 mL/min. To identify and quantify the flavonoid compounds in the petals of *S. littorea* we performed multiple reactions monitoring (MRM) combined with precursor ions scan and subsequent MS/MS analysis (Li et al. 2006, Rak et al. 2010). We used the standards of the flavonoids that were previously reported for *S. littorea* and others

species of *Silene* (Additional file 3). The standards were obtained from SDS (Toulouse, France). The parameters for the MRM transitions and HPLC-ESI-MS/MS analyses were fixed following Dardanelli et al. (2008), with the exception of the dwell time for each transition was 0.05 s. Chromatogram area was corrected for the total area of the petals that was measured with the software ImageJ (US National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/). Data were standardized by their maximum value. Significant differences in individual flavonoid concentrations were examined for the three color categories (P, L and W) in an ANOVA after log transformation in R v.3.1.0 (R Core Team 2013). When significant, we conducted Tukey HSD *post-hoc* paired tests to determine which color morphs exhibited significantly different mean flavonoid concentrations.

RESULTS

De novo assembly of ABP-related genes

We identified all 15 ABP-related genes from *de novo* assembly of the petal transcriptome. The longest contigs from the *de novo* assembly were most frequently from Velvet *k*-mer 31, but supplemented by contigs from other kmer analyses as necessary. After BLAST+ identification against the *A. thaliana* RefSeq, multiple putative paralogues were identified for seven genes producing a total of 29 ABP-related loci (Table 1). Three of the genes that feed into the ABP had two or three copies each (*Pal, C4h, and 4Cl*). Most of the ABP structural genes and their side-branches had only a single locus expressed in the petals except *Chs* and *F3h* which had two and three copies respectively. Of the three regulatory loci, there were six *Myb* isoforms, two *Wd40* isoforms and one for the *Bhlh*. We sequenced 100% of the coding sequence (CDS) for 28 of the 29 ABP-related loci. The CDS of one *Myb*

regulatory locus was only partially sequenced (*Myb6*). In addition, we acquired an average of 121 bp of the 5' UTR sequence (range 35-451bp) and 170 bp of the 3' UTR (range 21-306) (Table 1).

Sequence comparisons among color morphs

We found a total of 622 SNPs for the 29 ABP-related loci including the 5' UTR, CDS and the 3' UTR (Table 1). The number of SNPs per gene was highly variable, ranging from zero to 91 SNPs in *F3h1* and *Pal1*, respectively (Table 1). Although we found numerous non-synonymous SNPs in several loci, none of them consistently differentiated the three color morphs.

Ans was the only gene that had SNPs with allele frequencies consistently associated with flower color (Additional file 4). A total of 32 SNPs were found in the 5'UTR, CDS and 3' UTR in *Ans*, yet nine of these between positions 697-1099 exhibited substantially different frequencies in dark pink versus white samples (Figure 2). Furthermore, the likelihood of being homozygous for one allele or the other was also strongly correlated with petal color (Additional file 5). There was a very low likelihood of heterozygosity at all nine of these SNPs. For most of these SNPs, the intermediate, light pink individuals were assigned to either homozygote genotype and unlikely the heterozygous genotype (Additional file 5). Nevertheless, all of these color-differentiating SNPs were synonymous.

Table 1. Summary of length and number of single nucleotide polymorphisms (SNPs) in the 5' UTR, CDS and 3' UTR and number of non-synonymous SNPs for each locus. The complete CDS was found for all the genes shown except for one Myb regulator.

Locus	5' UTR length (bp)	CDS length (bp)	3' UTR length (bp)	#SNPs in 5' UTR	#SNPs in CDS	#SNPs in 3' UTR	Total # SNPs	# Non-synonymous SNPs
Pal1	119	2154	234	6	75	10	91	7
Pal2	118	2106	221	0	34	5	39	3
Pal3	55	2203	84	0	27	0	27	11
C4h1	78	1521	237	1	11	2	14	2
C4h2	451	1191	21	4	10	1	15	1
4Cl1	88	1692	147	1	27	2	30	4
4Cl2	99	1677	75	1	37	1	39	5
Chs1	60	1176	87	0	38	1	39	1
Chs2	136	1176	169	0	2	0	2	1
Chi	114	717	279	0	11	1	12	1
F3h1	129	1098	198	0	0	0	0	-
F3h2	179	1083	105	0	1	0	1	
F3h3	73	1050	114	1	25	1	27	
Dfr	108	1059	207	1	7	2	10	1
Ans	117	1098	216	2	24	6	32	7
Uf3gt	78	1374	273	1	28	5	34	8
At	88	1494	189	3	21	3	27	5
F3'h	41	1539	306	0	36	3	39	5
Fls	157	1176	270	1	3	0	4	2
Myb1a	128	708	160	1	10	2	13	1
My1b	61	729	152	0	4	2	6	2
Myb2	160	879	141	0	19	1	20	1
Myb3	222	711	147	1	14	2	17	4
Myb4	113	1032	72	0	5	1	6	3
Myb6	190	577*	-	0	5	-	5	0
Myb7	143	750	94	0	9	1	10	4
Wd401	106	1053	306	2	9	4	15	2
Wd402	35	1023	60	0	1	0	1	0
Bhlh	54	1914	198	1	42	4	47	24

* Partial coding sequence

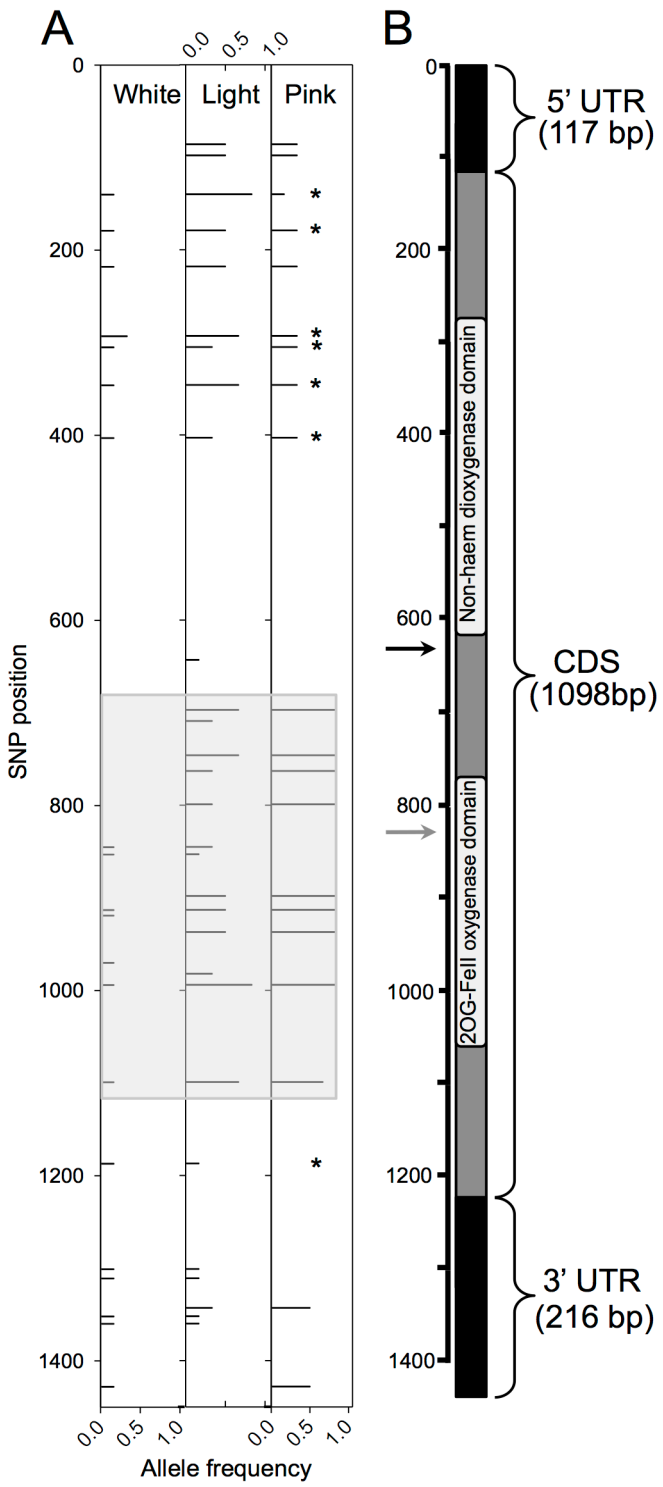

Figure 2. Allele frequencies of the SNPs in *Ans* (A) relative to the main functional domains of the gene (B). In (A) grey box highlights the zone where the nine synonimous SNPs that correlate with flower color are located (between bp 697 and 1100). Asterisks indicate non-synonymous SNPs. In (B), the gene is composed of a 5' UTR, 3' UTR and CDS containing two functional domains. The location of the intron is inferred from alignment with genomic DNA from other model species is indicated with a black arrow in (B). The grey arrow indicate the inferred position of the iron binding domain.

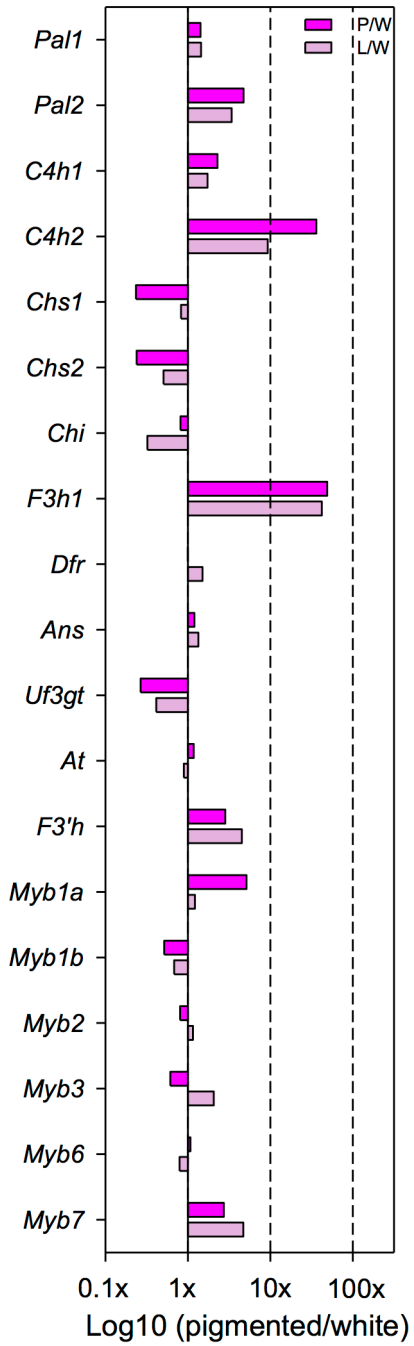

Figure 3. Expression differences, estimated as fold changes, between pink/white (pink bars) and light/white (light pink bars) petals at bud developmental stage.

Expression comparisons among color morphs

Since petal anthocyanins were phenotypically detected in the bud stage, we infer that all ABP-related loci should have been expressed by this developmental stage (Additional file 1). Thus, we focus on the three bud stage samples for expression comparisons (expression values for later developmental stages are available in Additional file 6). The DESeq corrected expression estimates for the bud stage ranged from 1,459.8 reads – 494,875.6 reads (median = 8,713.9 reads; Additional file 6).

When comparing dark pink to white petal buds, there are three loci with significantly higher expression in dark pink than white: *F3h1* (P/W = 49.0x; p = 0.039), *C4h2* (P/W = 36.2x; p = 0.013), and *Myb1a* (P/W = 5.1x; p = 0.009) (Figure 3, Additional file 6). When comparing light pink to white petal buds, there are two significantly differentiallly expressed loci: *F3h1* (L/W = 42.2x; p = 0.049) and *F3'h* (L/W = 4.5x; p = 0.047). Chalcone isomerase (*Chi*) is the only locus with W > L, yet only weakly so (L/W = 0.32x; p = 0.055 (Figure 3, Additional file 6).

When comparing the two pigmented morphs (dark pink and light pink), there are two signficantly differentially expressed loci – *Myb1a* (P/L = 4.2x; p = 0.021) and *Myb3* (P/L = 0.3; p = 0.033) (Additional file 6).

Phylogenetic analysis of R2R3 *Myb* loci

The phylogenetic analysis of the R2R3 *Myb* DNA binding domain including several ABP-related *Myb*s from model species indicates numerous *S. littorea Myb*s are likely ABP regulators. In particular, *SlMyb6* and *SlMyb7* grouped with a large number of unresolved *Mybs* from across the eudicots from subfamily 6 which are known to control the later steps of the ABP (Figure 4; Dubos et al. 2010). Within this largely unresolved polytomy, *SlMyb7* is weakly supported to be related to AtMyb75

and AtMyb90 also known as *PAP1* and *PAP2* because they control flavonol biosynthesis in vegetative tissues (Gonzalez et al. 2008). Less likely related to the ABP, *SlMyb2* and *SlMyb3* grouped in a clade with other non-anthocyanin regulators from subgroup 4 of *Arabidopsis thaliana* (Dubos et al. 2010). Similarly, *SlMyb1a* and *SlMyb1b* are supported as sister to *AtMyb4* (a repressor of the expression of *C4h*; Jin et al. 2000), however distantly related.

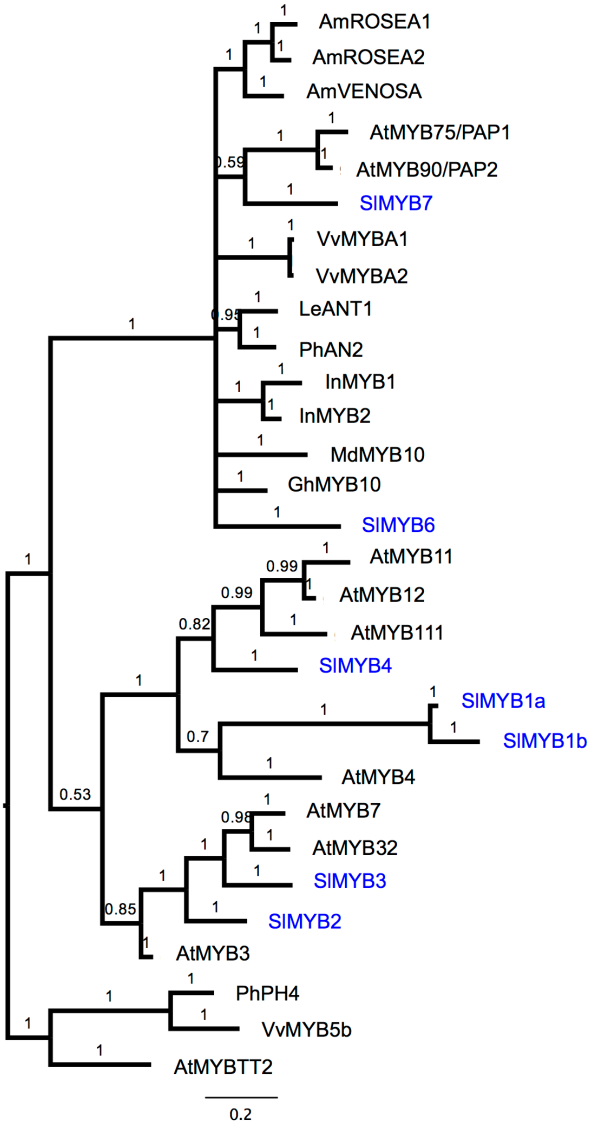

Figure 4. Phylogenetic analysis of *Myb* R2R3 DNA binding domains for *S. littorea* and other model species. The R2R3 domains of six Mybs identified in the *S. littorea* petal transcriptome (highlighted in blue) were aligned and analyzed under Bayesian phylogenetic methods. Posterior probabilities greater than 0.5 are indicated at the nodes. Branches are drawn proportional to the number of substitions per site (see scale bar). Species abbreviations: At: *Arabidopsis thaliana*, Am: *Antirrhinum majus*, Gh: *Gerbera hybrida,* In: *Ipomoea nil*, Le: *Lycopersicon esculentum*, Md: *Malus domestica*, Ph: *Petunia hybrida*, Sl: *Silene littorea*, Vv: *Vitis vinifera*. Genbank accession numbers can be found in Additional file 2.

Identification and quantification of flavonoids

HPLC analysis revealed three anthocyanin compounds (glycosylated cyanidin derivatives) responsible for the petal color in *S. littorea* (Additional file 7). We detected seven additional flavonoids: four flavones (identified from standards as apigenin, isoorientin, isovitexin and luteolin), two flavonols (quercetin and rutin), and one dihydroflavonol (dihydroquercetin). The putative location of these flavonoid intermediates in the ABP is shown in Figure 5.

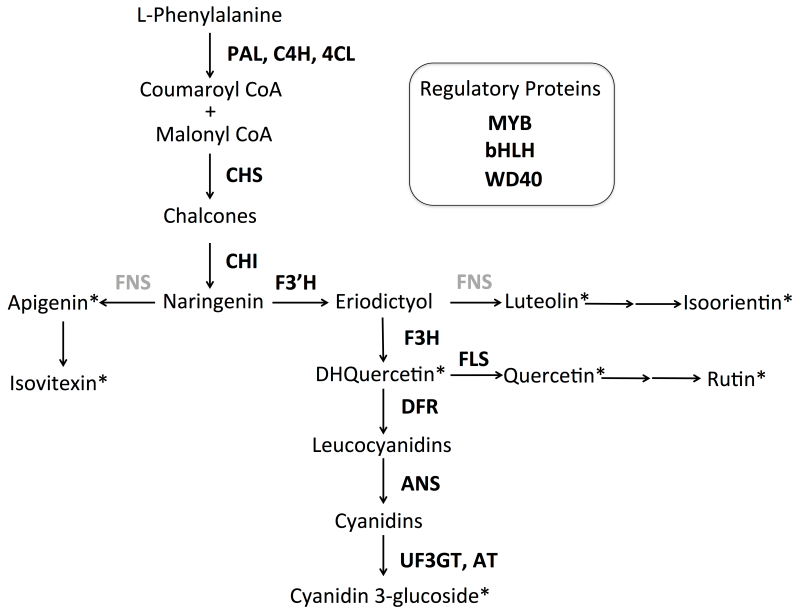

Figure 5. Tentative anthocyanin biosynthetic pathway for *S. littorea*. The pathway consists of a primarily linear pathway with three side branches that produce the anthocyanin pigment cyanidin. Flavonoids detected by HPLC are indicated with asterisks. Enzymes not included in this study are indicated in grey. Enzyme abbreviations are indicated next to arrows: PAL: phenylalanil ammonia-lyase; C4H: cinnamic acid 4-hydroxylase; 4CL: coumarate CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; FNS: flavone synthase; F3'H: flavonoid 3'hydroxylase; F3H: flavanone-3-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; UF3GT: glycosyl transferase; AT: acyltransferase. The three gene regulatory complex consists of a basic Helix Loop Helix protein (bHLH), WD Repeats (WD40) and R2R3-MYB domains (MYB) in most angiosperms.

The relative amounts of anthocyanins and their intermediates compared across color morphs can be used to biochemically relate the transcriptome results to the phenotype. The amount of cyanidin derivatives significantly increased with the

intensity of the petal color as expected (Table 2, Figure 6). In three of the five flavonoid intermediates (rutin, isovitexin, and quercetin), the relative amounts of metabolites in the color morphs were significantly different. Relative amounts of luteolin derivatives and apigenin were not significantly different among the color morphs (Table 2, Figure 6). *Post-hoc* pairwise comparisons among the three color morphs indicate that differences were always found between pigmented and white petals, except for quercetin where differences between white and pink were not significant. Light and dark pigmented morphs did not differ in the relative amount of any of the five flavonoid intermediates (Table 2, Figure 6).

Table 2. Statistical analysis of petal flavonoid concentrations. ANOVA results and pairwise Tukey *post-hoc* analyses for significant differences of flavonoid concentrations among the three color morphs (dark pink, light pink and white). Tukey *post-hoc* tests were only performed on flavonoids with significant ANOVA results and significant Tukey post-hoc results ($p < 0.05$) are indicated in bold.

Flavonoid	ANOVA	Pairwise Tukey *post-hoc* p-values		
	F-statistics	White - Light	White - Pink	Light - Pink
Cyanidin[a]	14.18***	**0.046**	**0.001**	**0.024**
Rutin	19.71***	**0.001**	**0.002**	0.120
Quercetin	5.557*	**0.015**	0.108	0.390
Luteolin[b]	3.098	-	-	-
Apigenin	3.722	-	-	-
Isovitexin	10.25**	**0.002**	**0.022**	0.243

[a] The three cyanidin derivatives were pooled in the ANOVA because our MS/MS quantification experiments do not allow differentiating between them. [b] Luteolin and isoorientin (luteolin hexoside) were pooled for the same reason.*P<0.05; **P<0.01; ***P<0.001

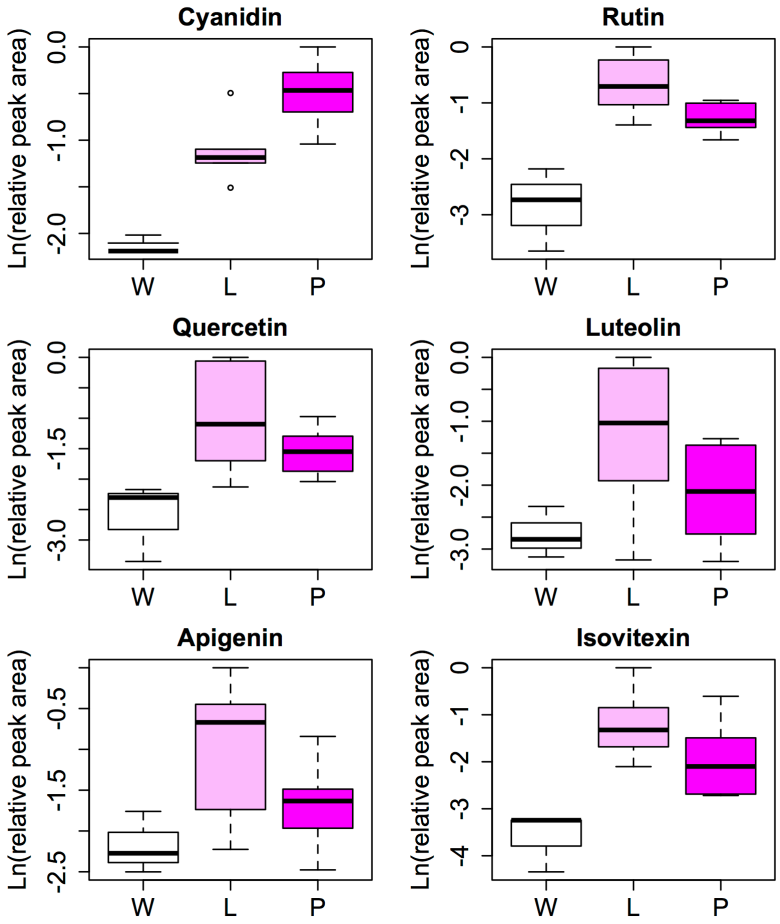

Figure 6. Comparison of flavonoid concentrations among color morphs. Six classes of flavonoids were identified by HPLC and labeled above each box plot. Cyanidin is the primary anthocyanin pigment. The remaining five flavonoids are intermediates in the pathway (see Figure 5). Log transformed relative peak areas are compared for white (W), light pink (L) and dark pink (P) samples. The boxes represent the 25th and 75th percentiles, the whiskers are the 5th and 95th percentiles, the central solid lines are the median values, and circles represents outliers.

DISCUSSION

We sequenced and measured expression of all ABP-related genes in the petal of the non-model species, *S. littorea*. We assembled complete coding sequences of 28 out of 29 ABP-related loci and identified over 600 SNPs, yet none are sufficient to confer a structural blockage in the ABP. This study is the first to sequence and measure expression of structural and regulatory genes of the ABP in the petals of *Silene*. A previous transcriptome analysis of white flowered *S. vulgaris* has been completed, but it utilized pooled RNA from leaves, roots and whole flowers (Sloan et

al. 2012, Blavet et al. 2011). Recently, RNA-Seq studies in non-model species of *Mimulus*, *Muscari* and *Parrya* have used *de novo* assemblies followed by expression analyses of the ABP genes (Yuan et al. 2014, Lou et al. 2014, Butler et al. 2014). Like our study, most of these identified the ABP genes and some of the regulatory transcription factors, confirming RNA-Seq as an efficient tool when a reference genome is not available. We assembled the complete CDS of most of the ABP genes (28 out of 29) compared to an average of 71% and 89% in *Muscari* and *Parrya*, respectively (Butler et al. 2014, Lou et al. 2014).

The large number of tightly clustered synonymous color-differentiating SNPs found in the coding sequence of *Ans* is unique and warrants further investigation. In *A. thaliana*, *Ans* contains two conserved domains: a non-haem dioxygenase domain between positions 274-616 and a 2OG-FeII oxygenase domain between positions 766-1057. These two functional domains are separated by a small intron that would start after *S. littorea* ANS 634 bp and continue for approximately 84 bp (the length in *A. thaliana* NC_003075.7). All of the color-differentiating SNPs in *S. littorea* were located after the intron, many of them in the 2OG-FeII oxygenase domain (Figure 2B), where a Fe binding site is predicted (Figure 2B)(Wilmouth et al. 2002). There are non-color differentiating SNPs on either side of this cluster of interesting SNPs. These could be indicative of a color-differentiating regulatory element in the intron, yet this has never been reported for ANS. However, none of these SNPs cause any changes to the amino acids, thus no changes in the protein activity are expected. Furthermore, in *Arabidopsis* and *Ipomoea*, the regulation of *Ans* occurs in the promoter (Xu et al. 2014, Dong et al. 2014), where MYB and bHLH binding sites are found. Further investigation of the intron sequence using a larger sampling is required to determine the significance of this encouraging finding.

The expression analysis identified several significantly differentially expressed genes with substantially decreased expression in white samples compared to pigmented samples. In particular, *F3h* exhibits significantly different expression for both dark pink vs. white and light pink vs. white comparisons. In fact, these are the two largest fold-changes in expression of pigmented versus white. Structural mutations can be discarded since no SNPs have been found in the coding region of *F3h*; thus, changes in expression could be due to *cis*-regulatory elements in the promoter or introns. Unfortunately, the lack of variation extends into the immediately upstream 5' UTR portion obscuring our ability to associate this region or a nearby region with any color-differentiating SNPs. Mutations upstream from the 5'UTR cannot be excluded. Mutations in *F3h* affecting flower color have been reported in various species in the literature (Dedio et al. 1995, van Houwelingen et al. 1998), indicating that this gene alone can be responsible of color changes. Nishihara et al. (2014) found that a colorless phenotype in *Torenia* was caused by a retrotransposon in the promoter of the *F3h* gene. In addition, antisense suppression of *F3h* in carnation resulted in a variety of transgenic plants showing a range of orange coloration, from attenuation to complete loss of the original orange color (Zuker et al. 2002). The regulation of ABP genes through changes in expression of their regulatory elements, could also lead to the differential expression observed in *F3h*. In *Mimulus aurantiacus*, *MaMyb2* regulates the expression of *F3h*, *Dfr*, and *Ans*; when *MaMyyb2* was silenced, the expression of these genes was significantly lower than the control (Streisfeld et al. 2013). In *S. littorea,* the gene tree of MYBs, placed *SlMyb6 and SlMyb7* in the same group as *MaMyb2* (data not shown) and many other known ABP regulators, and also presented a reduction of expression in the non-anthocyanin morph, although not significant. This result highlight *SlMyb6* and *SlMyb7* as a

tentative regulators of the expression of the ABP genes, however further experiments are needed to test this hypothesis.

Significant differences in expression were also found in *C4h2* (dark pink vs. white), *F3'h* (light pink vs. white) and the transcription factor *Myb1a* (dark pink vs. white and dark pink vs. light pink). *C4h2* is a pre-ABP gene acting in the general phenylpropanoid route (Ehlting et al. 2006). Suppression of early genes of ABP as *Chs* or *Chi* allows eliminating most of flavonoids without affect the production of other compounds of important side biosynthetic branches, as those of volatile benzenoids responsible for floral scent (Clark and Verwoerd 2011). This is because *Chs* or *Chi,* and also *C4h2*, are located downstream of the cinnamic acid, the initial substrate of this side branch (Davies et al. 2006, Zvi et al. 2008). The fact that we have found that white petals have low levels of all flavonoid intermediates, suggest that we cannot discard that changes in expression of the *C4h2* is downregulating the ABP in the white morph. In fact, although differences were not significant, expression of *C4h2* were also much higher in light vs. white morphs. The phylogenetic tree of Mybs, have shown that *Myb1a* (closely related to *Myb1b*, with a 68% of aminoacid similarity) is distantly related to *AtMyb4*, a known suppressor of *C4h* (Jin et al. 2000). On the other hand, we also found that light pink and dark pink petals showed differential expression in two *Myb* transcriptional regulators. This suggests that differences in color intensity between the light and dark pink morphs could be due to a differential regulation of any candidate ABP genes (e.g. Hopkins and Rausher 2011, Yuan et al. 2013), rather than heterozygosity of a single loss-of-function locus in the white morph. In fact, during the SNP assignment, light pink morph was not related with a higher probability of being heterozygote. However this needs to be evaluated with a F_2 segregation experiment.

A structural or regulatory blockage in the ABP decrease the amount of flavonoid intermediates below the blockage, but would increase the amount of intermediates in upstream side branches (depending on the dynamics of metabolite flux through the pathway). The flavonoid biochemical analysis identified cyanidin as the primary anthocyanin and an additional five flavonoid intermediates to compare among the color morphs. Only three of them were significantly different between white and pigmented individuals and two (quercetin and rutin) are consistent with a blockage at or above F3H. Consistent results between HPLC and expression analysis were found in *Parrya nudicaulis*, where the white morph did not produce chatequins or flavonols due to the reduced expression in *Chs* (Dick et al. 2011). Nevertheless, in *Iris lutescens* the production of non-anthocyanic flavonoids (including chalcones, flavones and flavonols) in the yellow morph was higher than in the purple (Wang et al. 2013). In *Muscari armeniacum,* Lou et al. (2014) found that for anthocyanins (delphinidin and cyanidin), the white morph contained the same metabolites as the blue, and generally at higher concentrations. They argue that the blockage in DFR in the white morph, produce a redirection of the flux of metabolites through the production of other intermediate metabolites. A similar argument may hold between the light and pink morphs in *S. littorea*. Although differences were not significant, the light morph of *S. littorea* showed a trend to higher concentrations of flavonols and flavones in the dark compared with the light morph (Figure 6), which could be due to a redirection of the flux of metabolites.

Based in our biochemical analysis we have proposed a tentative metabolic pathway of anthocyanin in the petals of *S. littorea* (Figure 5). The pink color of the petals is caused by the accumulation of cyanidin 3-gucoside derivatives, as is found in *S. armeria* (Iwashina et al. 1987) and *S. dioica* (Kamsteeg et al. 1979). Dark pink

flowers in *S. littorea* showed the same cyanidin 3-glucoside derivatives but in a much higher concentration than light pink flowers, which suggest that the pink intensity is caused by the different concentration of these compounds. In other species, it has been proposed that copigments such as flavones and flavonols play an important role in the color or intensity of the petals (Gould and Lister 2006, Thill et al. 2012, Nishihara et al. 2014). For example, brown color of outer part of the labelum of *Ophrys speculum* is suggested to be caused by the flavonols acting as copigments of cyanidins (Vignolini et al. 2012). In *S. littorea,* flavonols and flavones is unexpected that play a key role in the pink intensity because higher concentrations was not found in darker petals.

The lack of anthocyanins and the lower levels of other flavonoids in petals of the white-flowered morph of *S. littorea* could result in a fitness disadvantage due to their role in the interaction with pollinators, florivores or pathogens (e.g. Hoballah et al. 2007, Johnson et al. 2008, Falcone Ferreyra et al. 2012), and the tolerance against other abiotic variables (reviewed in Winkel-Shirley 2002). Interestingly, individuals of the white campion, *S. pratensis*, lacking glyscosilated isovitexin showed ruptured upper epidermal cells that caused curved petals (van Brederode et al. 1982). The possible disadvantage of the lack of anthocyanins or other flavonoids can be even higher when vegetative tissues are also affected (Levin and Brack 1995, Warren and Mackenzie 2001). This could be the case of a different type of white-flowered mutant that appear in some southern populations. This mutant is not able to produce anthocyanin in other tissues of the plant (see the calyx in Additional file 1E), and is found at very low frequencies (<0.05%; Casimiro-Soriguer et al. unpublished results). Functional mutations are commonly responsible of the spontaneous white-flowered mutants that appear at low frequencies in most of the species (i.e. Coberly and

Rausher 2008, Wu et al. 2013). Thus, the rare mutant in *S. littorea* could be also due to a coding mutation, but future experiments should be carried out to answer this question. However, the high frequent white-flowered mutant studied here, is able to produce anthocyanins and flavonoids in other tissues of the plant as calyx, leaves or steam (see calyx in Additional file 1D), and *cis*-regulatory mutations are the most probable cause of the lack of anthocyanins in this white-flowered morph.

CONCLUSIONS

We have used RNA-Seq to simultaneously sequence and estimate expression of 29 ABP-related loci among three flower color morphs of the non-model plant, *S. littorea*. After sequencing the complete coding sequences of all structural genes and most regulatory loci, we found a cluster of nine synonymous SNPs around the intron in *Ans* whose frequencies correlate with flower color, yet their functional significance is unclear. For expression, we see consistent significant downregulation in *F3h* when comparing pigmented and white petal buds. The flavonoid biochemical analysis is partially consistent with downregulation of *F3h* – the most likely candidate for the loss of floral anthocyanins among the 29 ABP-related loci identified herein. However, further genetic analysis of these color-differentiating results in a segregating F2 population is an essential next step in testing these newfound candidates.

AUTHORS' CONTRIBUTIONS

JBW, MLB, EN and ICS conceived and designed the experiments. MLB, JCdV, EN and ICS carried out the sampling. JBW and ICS performed the assembly, the sequences comparison and the phylogenetic analysis. MLB and ICS carried out the differential expression analysis. EN, JCdV and ICS analyzed the HPLC data.

JBW, EN, MLB and ICS drafted the manuscript. All authors read and approved the final manuscript.

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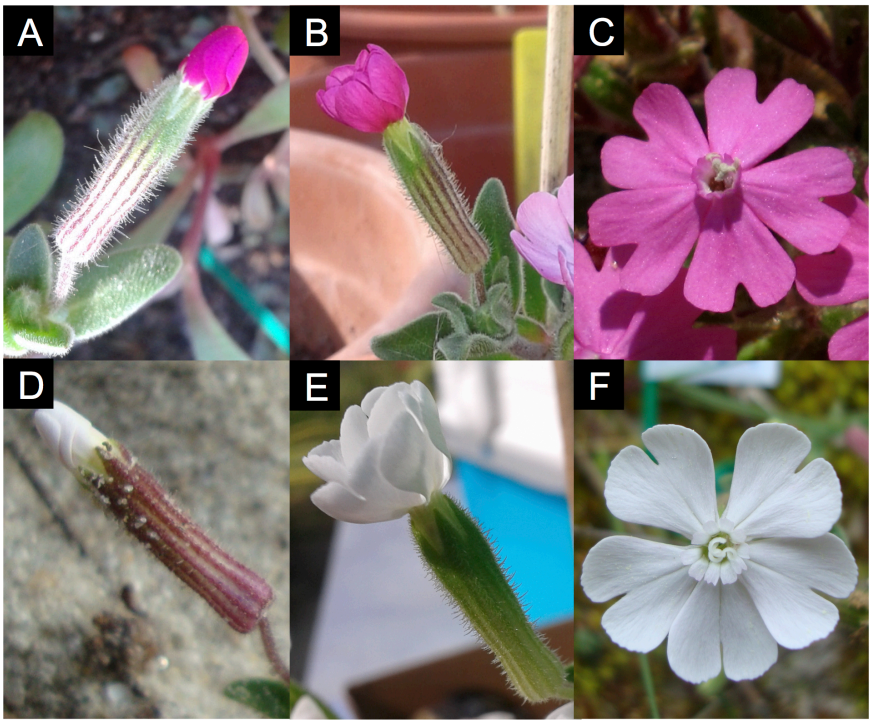
SUPPORTING INFORMATION

Additional file 1. Developmental differences in dark pink and white petals. Images of the three developmental stages sampled for dark pink and white flower color morphs of Silene littorea: bud (A, D), opening (B, E), and anthesis (C, F).



Additional file 2. Accession numbers for R2R3 *Myb* phylogenetic analysis. This table provides the species, gene name and Genbank accession numbers for the samples used in the phylogenetic analysis of *S. littorea* R2R3 *Mybs*.

Taxon	Gene Name	Genbank Accession #
Antirrhinum majus	AmROSEA1	DQ275529
	AmROSEA2	DQ275530
	AmVENOSA	DQ275531
Arabidopsis thaliana	AtMYBTT2	NM_122946
	AtMYB4	AB005889
	AtMYB111	NM_124310
	AtMYB12	NM_130314
	AtMYB11	NM_116126
	AtMYB3	NM_102111
	AtMYB7	NM_127224
	AtMYB32	NM_119665
	AtMYB75/PAP1	NM_104541
	AtMYB90/PAP2	NM_105310
Gerbera hybrida	GhMYB10	AJ554700
Ipomoea nil	InMYB1	AB232770
	InMYB2	AB234211
Lycopersicon esculentum	LeANT1	AY348870
Malus domestica	MdMYB10	AB744002
Oryza sativa	OsMYB4	D88620
Petunia hybrida	PhPH4	AY973324
	PhAN2	AF146702
Vitis vinifera	VvMYB5b	AY899404
	VvMYBA1	XM_003631456
	VvMYBA2	AB097924
Zea mays	ZmP1	L19495
	ZmC1	AF320614

Additional file 3. Flavonoid standards for HPLC analysis. Standards used for identifying and quantifying flavonoids in the multiple reaction monitoring experiments, their mass transitions, and the references in which the compound derivatives are found in other *Silene* species.

Flavonoid Type	Standard	Mass transitions	References
Anthocyanin	Peonidin	301.1/301.1	1 (P)
Anthocyanin	Pelargonidin	271.1/271.1	2 (P)
Anthocyanin	Cyanidin[a]	287.1/287.1	1, 2 (P)
Flavanone	Naringenin	271.0/119.0	-
Flavone	Apigenin[a]	269.0/117.0	3, 4, 5, 6, 7 (P, L)
Flavone	Apigenin 7-*O*-glucoside[a]	431.0/268.0	3, 4, 5, 6, 7 (P, L)
Flavone	Luteolin[a]	285.0/133.0	3, 5, 7 (P, L)
Flavonol	Kaempferol	285.0/117.0	3, 4 (L)
Flavonol	Quercetin[a]	301.0/151.0	3, 4 (L)
Flavonol	Quercetin 3-*O*-rutinoside[a,c]	609.0/300.0	-
Flavanonols	Dihydroquercetin[b,c]	303.0/303.0	-
Isoflavone	Genisteine	269.0/133.0	4 (L)

[a] compound derivative was confirmed in *S. littorea*
[b] compound was only observed as trace in *S. littorea*
[c] compound first time confirmed in species of *Silene*

Table References

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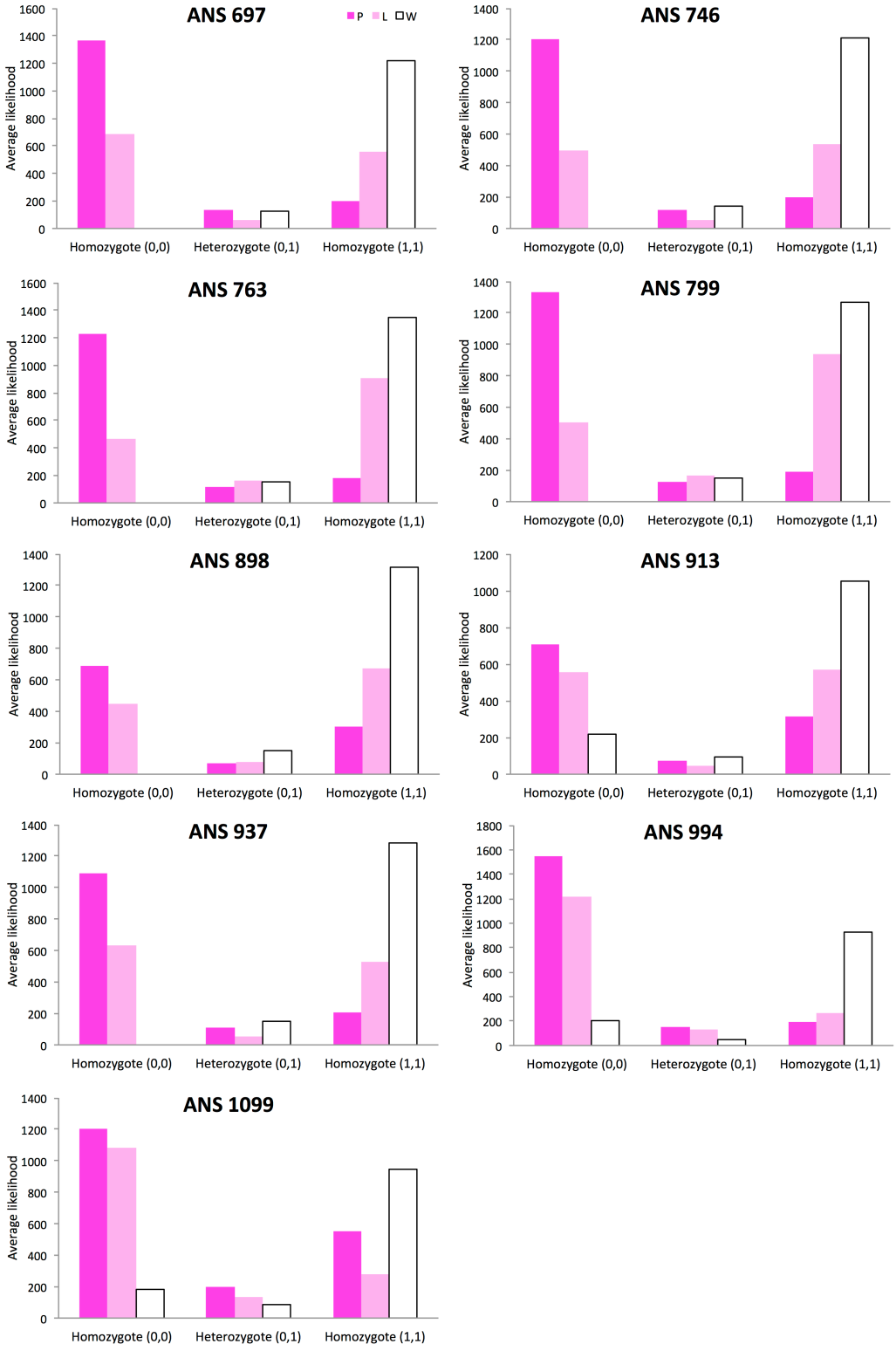
Additional file 4. Ans SNP characterization. Characteristics of the 32 SNPs found in the 5'UTR, the coding sequence (CDS) and 3' UTR of *Ans*. SNPs with color-differentiating allele frequencies are indicated in bold. W = white , L = light pink, P = dark pink.

Gene region	Alignment position	Reference allele	Alternate allele	Synonimous (S) or Non-synonimous (NS)	Alternate allele frequency in W	Alternate allele frequency in L	Alternate allele frequency in P
5' UTR	86	G	A		0.000	0.500	0.333
5' UTR	98	A	C		0.000	0.500	0.333
CDS	140	G	A,C	NS	0.167	0.833	0.167
CDS	179	C	G	NS	0.167	0.500	0.333
CDS	218	C	T	S	0.167	0.500	0.333
CDS	293	G	C	NS	0.333	0.667	0.333
CDS	305	A	G	NS	0.167	0.333	0.333
CDS	346	C	G	NS	0.167	0.667	0.333
CDS	403	G	C	NS	0.167	0.333	0.333
CDS	643	T	C	S	0.000	0.167	0.000
CDS	**697**	**A**	**C**	**S**	**0.000**	**0.667**	**0.833**
CDS	709	A	G	S	0.000	0.333	0.000
CDS	**746**	**C**	**T**	**S**	**0.000**	**0.667**	**0.833**
CDS	**763**	**C**	**A**	**S**	**0.000**	**0.333**	**0.833**
CDS	**799**	**G**	**A**	**S**	**0.000**	**0.333**	**0.833**
CDS	845	C	T	S	0.167	0.333	0.000
CDS	853	T	C	S	0.167	0.167	0.000
CDS	**898**	**C**	**G**	**S**	**0.000**	**0.500**	**0.833**
CDS	**913**	**A**	**G**	**S**	**0.167**	**0.500**	**0.833**
CDS	919	C	T	S	0.167	0.000	0.000
CDS	**937**	**C**	**A**	**S**	**0.000**	**0.500**	**0.833**
CDS	970	T	C	S	0.167	0.000	0.000
CDS	982	C	T	S	0.000	0.333	0.000
CDS	**994**	**G**	**T**	**S**	**0.167**	**0.833**	**0.833**
CDS	**1099**	**A**	**G**	**S**	**0.167**	**0.667**	**0.667**
CDS	1187	C	T	NS	0.167	0.167	0.000
3' UTR	1301	T	C		0.167	0.167	0.000
3' UTR	1311	C	T		0.167	0.167	0.000
3' UTR	1343	C	G		0.000	0.333	0.500
3' UTR	1352	T	C		0.167	0.167	0.000
3' UTR	1360	A	T		0.167	0.167	0.000
3' UTR	1428	T	A		0.167	0.000	0.500

Additional file 5. Genotype likelihoods for the nine synonymous SNPs in *Ans* that correlate with flower color. The likelihood of genotype assignments for homozygotes for the reference allele (0,0), homozygotes for the alternate allele (1,1) and heterozygotes (0,1) for dark pink (P), light pink (L) and white (W) samples from GATK. Alignment position is indicated above each graph.



Additional file 6. Expression differences between color morphs across developmental stages. Expression corrected values and pairwise fold-change comparisons among color morphs and developmental stages for the 29 ABP-related loci.

BUD EXPRESSION						
	Corrected expression values			Fold change		
Loci	Pink	Light	White	P/W	L/W	P/L
Pal1	11227.9	11376.8	7880.8	1.4	1.4	1.0
Pal2	494875.6	354991.8	104448.7	4.7	3.4	1.4
Pal3	Filtered	Filtered	Filtered	-	-	-
C4h1	37635.7	28535.9	16466.5	2.3	1.7	1.3
C4h2	52874.9	13603.8	1459.8	**36.2**	9.3	3.9
4Cl1	Filtered	Filtered	Filtered	-	-	-
4Cl2	Filtered	Filtered	Filtered	-	-	-
Chs1	6094.3	21439.8	25948.1	0.2	0.8	0.3
Chs2	2087.1	4420.8	8713.9	0.2	0.5	0.5
Chi	10885.0	4319.7	13372.6	0.8	0.3	2.5
F3h1	73300.0	63067.7	1495.7	**49.0**	**42.2**	1.2
F3h2	Filtered	Filtered	Filtered	-	-	-
F3h3	Filtered	Filtered	Filtered	-	-	-
Dfr	8568.6	12976.5	8607.5	1.0	1.5	0.7
Ans	13372.4	14939.3	11138.7	1.2	1.3	0.9
Uf3gt	7360.6	11363.1	27497.6	0.3	0.4	0.6
At	8529.3	6460.1	7227.2	1.2	0.9	1.3
F3'h	5613.5	8889.9	1969.9	2.8	**4.5**	0.6
Fls	Filtered	Filtered	Filtered	-	-	-
Myb1a	21442.0	5085.6	4171.7	**5.1**	1.2	**4.2**
Myb1b	4394.4	5782.2	8496.0	0.5	0.7	0.8
Myb2	5423.4	7756.6	6723.5	0.8	1.2	0.7
Myb3	3152.2	10583.5	5140.7	0.6	2.1	**0.3**
Myb4	Filtered	Filtered	Filtered	-	-	-
Myb6	16931.9	12480.6	15756.5	1.1	0.8	1.4
Myb7	4175.2	7195.7	1530.3	2.7	4.7	0.6
Wd401	Filtered	Filtered	Filtered	-	-	-
Wd402	Filtered	Filtered	Filtered	-	-	-
Bhlh	Filtered	Filtered	Filtered	-	-	-
OPENING EXPRESSION						
	Corrected expression values			Fold change		
Loci	Pink	Light	White	P/W	L/W	L/P
Pal1	13899.1	17384.5	29031.4	0.5	0.6	0.8
Pal2	181519.5	70311.2	219069.5	0.8	0.3	2.6
Pal3	2781.2	574.1	5732.4	0.5	0.1	4.8
C4h1	54156.6	129645.7	131416.0	0.4	1.0	0.4
C4h2	11208.5	16123.7	5256.7	2.1	3.1	0.7
4Cl1	2048.3	13119.7	5439.1	0.4	2.4	0.2
4Cl2	Filtered	Filtered	Filtered	-	-	-
Chs1	64507.1	17417.9	95468.0	0.7	0.2	3.7
Chs2	6067.0	5249.6	2730.8	2.2	1.9	1.2
Chi	18093.4	7847.8	14507.5	1.2	0.5	2.3
F3h1	55358.2	108048.4	34653.6	1.6	3.1	0.5
F3h2	Filtered	Filtered	Filtered	-	-	-
F3h3	Filtered	Filtered	Filtered	-	-	-
Dfr	6176.6	7508.8	271.6	**22.7**	**27.6**	0.8
Ans	10488.3	4911.9	2507.3	4.2	2.0	2.1
Uf3gt	5816.1	2765.2	887.0	6.6	3.1	2.1
At	26617.0	12866.1	15538.9	1.7	0.8	2.1
F3'h	8397.1	8274.7	10390.8	0.8	0.8	1.0
Fls	Filtered	Filtered	Filtered	-	-	-
Myb1a	4084.0	10942.2	12233.0	0.3	0.9	0.4
Myb1b	Filtered	Filtered	Filtered	-	-	-
Myb2	5157.0	7605.3	3473.4	1.5	2.2	0.7

Myb3	Filtered	Filtered	Filtered	-	-	-
Myb4	Filtered	Filtered	Filtered	-	-	-
Myb5	Filtered	Filtered	Filtered	-	-	-
Myb6	9424.4	15265.1	14868.4	0.6	1.0	0.6
Myb7	6432.4	29972.2	19830.2	0.3	1.5	**0.2**
Wd401	Filtered	Filtered	Filtered	-	-	-
Wd402	Filtered	Filtered	Filtered	-	-	-
Bhlh	Filtered	Filtered	Filtered	-	-	-

ANTHESIS EXPRESSION

	Corrected expression values			**Fold change**		
Loci	**Pink**	**Light**	**White**	**P/W**	**L/W**	**P/L**
Pal1	19718.7	26183.0	30892.6	0.6	0.8	0.8
Pal2	244895.1	246964.6	159794.4	1.5	1.5	1.0
Pal3	3893.3	9419.8	2831.1	1.4	3.3	0.4
C4h1	124206.3	131175.3	129845.9	1.0	1.0	0.9
C4h2	14706.6	9413.2	8742.8	1.7	1.1	1.6
4Cl1	7593.1	3005.9	2701.1	2.8	1.1	2.5
4Cl2	Filtered	Filtered	Filtered	-	-	-
Chs1	49619.5	106225.9	16991.9	2.9	**6.3**	0.5
Chs2	4507.4	17097.7	1066.3	4.2	16.0	0.3
Chi	19249.6	22681.9	13365.0	1.4	1.7	0.8
F3h1	82269.0	34089.9	142819.6	0.6	0.2	2.4
F3h2	Filtered	Filtered	Filtered	-	-	-
F3h3	Filtered	Filtered	Filtered	-	-	-
Dfr	5457.7	5294.4	3419.4	1.6	1.5	1.0
Ans	13391.8	8980.8	9763.1	1.4	0.9	1.5
Uf3gt	6259.6	6572.6	2782.5	2.2	2.4	1.0
At	4257.1	2000.5	13966.4	0.3	0.1	2.1
F3'h	11068.4	17520.6	14428.6	0.8	1.2	0.6
Fls	Filtered	Filtered	Filtered	-	-	-
Myb1a	7899.3	8972.2	16273.6	0.5	0.6	0.9
Myb1b	Filtered	Filtered	Filtered	-	-	-
Myb2	2233.4	2706.6	6476.3	0.3	0.4	0.8
Myb3	Filtered	Filtered	Filtered	-	-	-
Myb4	Filtered	Filtered	Filtered	-	-	-
Myb5	Filtered	Filtered	Filtered	-	-	-
Myb6	12226.0	16661.5	10346.1	1.2	1.6	0.7
Myb7	4877.6	4534.1	25960.5	0.2	**0.2**	1.1
Wd401	Filtered	Filtered	Filtered	-	-	-
Wd402	Filtered	Filtered	Filtered	-	-	-
Bhlh	Filtered	Filtered	Filtered	-	-	-

Additional file 7. Flavonoids identification. Results from the HPLC–ESI-MS/MS biochemical analysis of petals with flavonoid identifications.

Flavonoid type	Flavonoid identification[b]	t_R (min)	Parent ions (*m/z*)	MS/MS (*m/z*)[a]
Anthocyanin	Cyanidin acetylrutinoside-glucoside	4.00	800	637/449/287
Anthocyanin	Cyanidin 3-*O*-rutinoside-5-*O*-glucoside	4.57	757	595/449/287
Anthocyanin	Cyanidin acetylrutinoside-glucoside	5.70	800	637/449/287
Dihydroflavonol	Dihydroquercetin[c]	10.80	303	303
Flavone	Isoorientin	6.00	447	285/133
Flavone	Isovitexin	9.97	431	240
Flavone	Luteolin	10.84	285	133
Flavone	Apigenin	11.30	269	117
Flavonol	Rutin	9.48	609	300
Flavonol	Quercetin	10.94	301	151

[a] MS analysis was acquired in positive mode for anthocyanins and in negative mode for non-anthocyanin flavonoids; [b] identification was based on retention time and comparisons of MS data with standards and values of previously reported flavonoids for *Silene* species (see Additional file 8 and the database http://metabolomics.jp); [c] this compound was observed as trace.

7

Capítulo 7

Discusión General y Conclusiones

DISCUSIÓN GENERAL

Transiciones evolutivas de sistemas sexuales en Silene

Como hemos mostrado a lo largo de los capítulos, la expresión sexual en *Silene* es muy variable tanto a nivel de género (Capítulo 2) como de especies (Capítulos 2 y 3), poblaciones (Capítulo 3) e individuos (Capítulo 5). Así, en *Silene* se ha encontrado que casi el 60% de las especies son hermafroditas, un 14% dioicas, un 13% ginodioicas y un 12% ginodioicas-ginomonoicas. Si bien es cierto que estas estimaciones están basadas en un 14% de las especies del género, a día de hoy es la información más actualizada y precisa. Hay que especificar que en nuestro estudio sólo hemos tenido en cuenta aquellas especies en las que el sistema sexual ha sido analizado en profundidad. De esta forma, se han evitado las descripciones de las floras generales (excepto casos puntuales como el de las especies dioicas o alguna ginodioicas que aparecen recogidas), ya que como hemos comprobado para el caso de la sección *Psammophilae*, la descripción de su sistema sexual era impreciso.

Actualmente, una reconstrucción en detalle de la historia evolutiva de los sistemas sexuales en *Silene* no es posible debido a (1) la falta de una filogenia bien resuelta, al menos hasta el nivel de sección, y (2) al bajo número de especies en las que se conoce con precisión el sistema sexual. Sin embargo, eso no ha impedido que se hayan podido extraer patrones a partir del estudio de una parte de las especies del género, y especialmente de los dos clados que incluyen a las especies dioicas (e.g. Marais et al. 2011, Slancarova et al. 2013). Aunque el estado ancestral del género no está claro, pudiendo ser hermafrodita o ginodioico (Desfeux et al. 1996, Marais et al. 2011), en el Capítulo 3 se ha mostrado que la presencia de los diferentes sistemas sexuales en los dos subgéneros (*Silene* y *Behenantha*) es indicativa de que algunos de

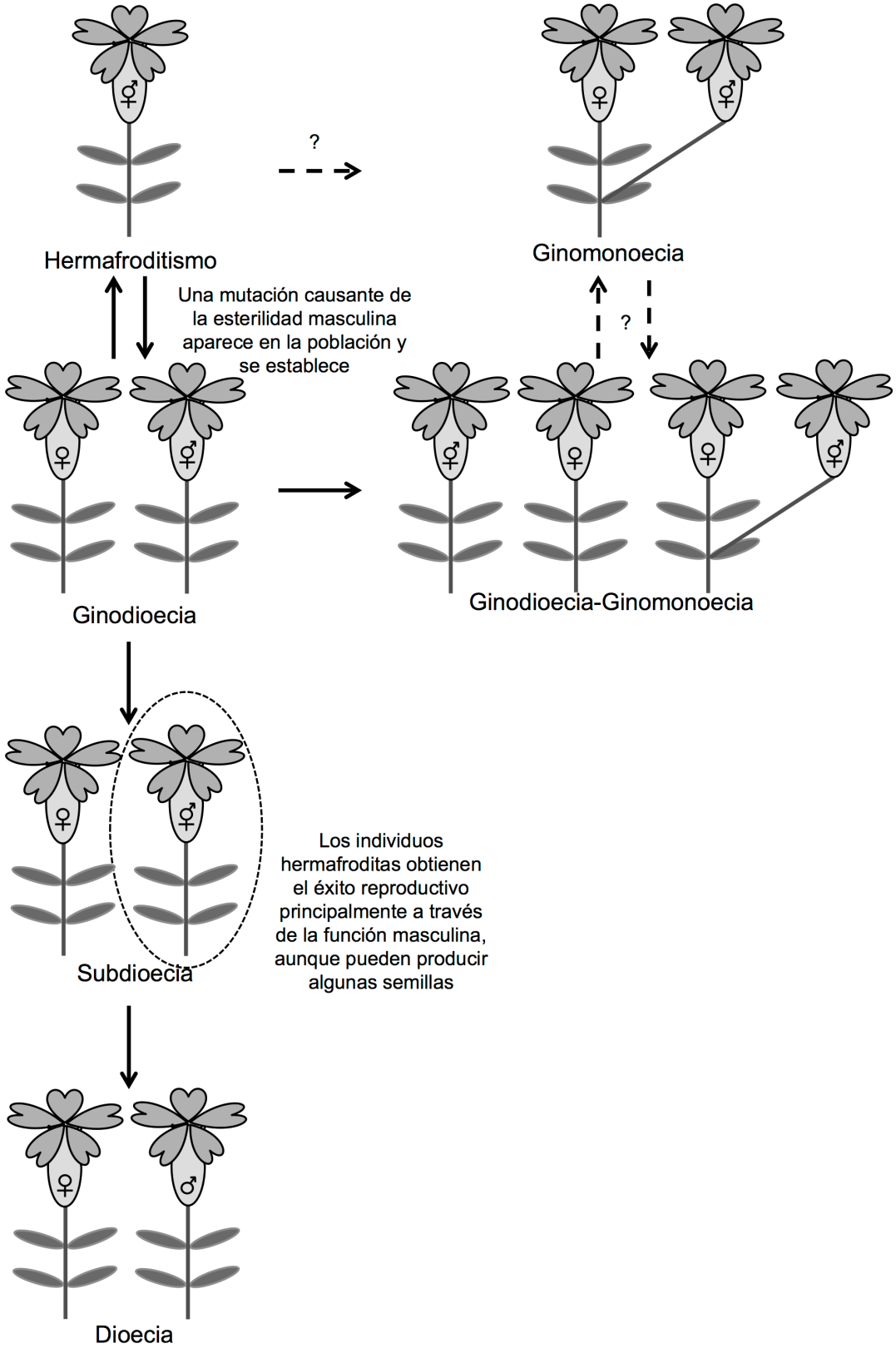


Figure 1. Sistemas sexuales en *Silene* y sus posibles transiciones evolutivas. Las flechas negras indican las transiciones más probables propuestas en la bibliografía y en la presente tesis doctoral, mientras que las flechas punteadas indican las transiciones menos probables.

ellos han surgido de forma independiente. Desfeux et al. (1996) ya apuntaban en esta dirección; ellos consideraron como estado ancestral la ginodioecia, y en base a ello encontraron que tanto el hermafroditismo como la dioecia habían surgido dos veces de forma independiente.

Existen varios artículos en los que se ha tratado la problemática de las posibles vías de evolución de los sistemas sexuales en *Silene* (Desfeux et al. 1996, Marais et al. 2011, Slancarova et al. 2013). En la Figura 1 se muestra un esquema de las transiciones más probables entre los sistemas sexuales más frecuentes en *Silene* realizado a partir de estos estudios. Además, incluimos nuestras aportaciones en el sistema sexual ginodioico-ginomonoico (Gd-Gm), cuyo origen más probable parece ser la ginodioecia, ya que desde el punto de vista genético, la Gd-Gm puede entenderse como una ginodioecia más compleja, en la que los individuos ginomonoicos presentan una restauración masculina incompleta (Koelewijn y Van Damme 1996) o bien presentan una mezcla de genomas mitocondriales que determinan si la flor es femenina o hermafrodita (Andersson 1999).

Únicamente hemos encontrado una especie ginomonoica (*S. noctiflora*, Capítulo 3), y su origen no está claro, pudiendo ser a través de la Gd-Gm o del hermafroditismo. Sin embargo, dado el número de especies descritas como H-Gm en la literatura (Jürgens et al. 2002, Capítulo 3), y la ausencia de especies ginodioicas o Gd-Gm donde se hayan encontrado poblaciones exclusivamente ginomonoicas, la transición más probable hacia la ginomonoecia parece ser a través del hermafroditismo. La ginomonoecia se ha considerado como una de las rutas posibles para la evolución hacia la monoecia (de Jong, 2008). Sin embargo, no hemos incluido esta transición en la Figura 1 debido a la ausencia de especies monoicas descritas en *Silene* (Desfeux et al. 1996, Jürgens et al. 2002, Capítulo 3). Las transiciones entre ginodioecia y hermafroditismo están indicadas

en ambos sentidos, puesto que se desconoce el estado ancestral y ambas son posibles (Marais et al. 2011). Hemos incluido la subdioecia como paso previo a la dioecia, ya que ha sido descrita en poblaciones de *Silene acaulis*, en las que individuos femeninos coexisten con individuos funcionalmente masculinos aunque algunos producen algunos frutos (Maurice 1999).

Mantenimiento del sistema sexual ginodioico-ginomonoico en las poblaciones

La mayoría de las poblaciones de las especies estudiadas de la sección *Psammophilae* presentan ginodioecia-ginomonoecia, aunque algunas de ellas sean exclusivamente ginodioicas (Capítulo 3). Por otra parte, la frecuencia de las plantas femeninas es bastante baja, siempre menor del 20% (Capítulo 3, Guitián y Medrano 2000), por lo que el primer paso en la evolución de la dioecia a través de la ginodioecia, es decir, la expansión de las plantas femeninas en las poblaciones, no parece que se esté cumpliendo en las especies estudiadas. Por lo tanto, esta prevalencia de las poblaciones Gd-Gm, junto con los bajos porcentajes de plantas femeninas, parecen ser indicativos de que se trata de un sistema estable, al menos en esta sección. Los resultados encontrados en el Capítulo 4 aportan una posible explicación para estos bajos porcentajes de plantas femeninas, ya que podrían ser debidos a que resultan poco atractivas para los polinizadores debido a su menor tamaño, y a que no reciben suficiente cantidad de polen para fecundar el elevado número de óvulos. En este capítulo se confirma que las flores femeninas son más pequeñas en todas las especies, y que la deposición de polen es mayor en las flores hermafroditas. También encontramos gradientes de selección positivos para el tamaño floral en la deposición y germinación de polen. Aunque el principio de Bateman (1948) establece que el éxito reproductor femenino está más limitado por los recursos que por las oportunidades de apareamiento, una menor atracción frente a los polinizadores podría resultar en un déficit de polen si el número de

óvulos es elevado (Van Etten y Chang 2014). De hecho, muchos autores han encontrado una mayor atracción de los polinizadores hacia las flores hermafroditas en detrimento de las flores femeninas (Delph y Lively 1992, Williams et al. 2000, Asikainen y Mutikainen 2005).

En coherencia con los resultados encontrados en el Capítulo 4, en el Capítulo 5 se encontró que aquellos individuos más femeninos no tenían un mayor éxito reproductivo, ni en el cuajado de frutos y semillas ni en el número total de semillas. En este capítulo la utilización de medidas continuas para la expresión sexual de los individuos (*temporal phenotypic gender* y *functional gender*) permitió considerar un continuo entre los individuos femeninos, ginomonoicos y hermafroditas. De esta forma, pudimos dotar de mayor poder estadístico a los análisis debido al bajo número de individuos que habría, por ejemplo, en la categoría de plantas femeninas (Dufay et al. 2010). Por tanto, no encontramos en las plantas femeninas una compensación en la fecundidad por la pérdida de la función masculina (Charlesworth y Charlesworth 1978, Bailey et al. 2003, Dufay y Billard 2012). Por otra parte, el mantenimiento de los individuos femeninos es posible incluso con una mínima ventaja cuando la esterilidad masculina se controla a través de genes citoplasmáticos, como parece ser en la mayor parte de las especies investigadas (Charlesworth y Laporte 1998, Vilas y García 2006, Lafuma y Maurice 2006, Garraud et al. 2011). Además, las semillas producidas por las plantas femeninas son producidas mediante xenogamia, lo que supone una ventaja en caso de depresión por endogamia. Aunque no hemos explorado esta opción en esta tesis, Vilas y García (2006) encontraron que la proporción de plantas femeninas en *S. littorea*, así como de flores femeninas en los individuos ginomonoicos, fue mayor en poblaciones procedentes de autopolinización que en poblaciones procedentes de polinización cruzada. Esto tuvo un efecto en el reclutamiento de la población, pudiendo

deberse a la depresión por endogamia y/o a la limitación de polen. También en la especie ginodioica-ginomonoica *S. nutans*, se ha demostrado que la mayor eficacia reproductiva de las plantas femeninas depende de si hay limitación de polen (Lahiani et al. 2015). Cuando hay limitación de polen, las plantas hermafroditas tienen la ventaja del aseguramiento reproductivo. Sólo cuando hay suficiente disponibilidad de polen, las semillas procedentes de polinización cruzada de las plantas femeninas estarán en ventaja con respecto a las de las plantas hermafroditas, en las que son mayores los niveles de autofecundación (tanto por autogamia como por geitonogamia). En la especie ginodioica *Daphne laureola*, en la que el porcentaje de plantas femeninas varió entre el 20-56% a lo largo de un gradiente altitudinal, Alonso (2005) encontró que las plantas femeninas tenían en general un menor éxito de polinización que las hermafroditas. Además, debido a la escasez de polinizadores en altitudes elevadas, esta desventaja en la polinización se vio más acuciada, a pesar de que la disponibilidad de polen era mayor debido a que la proporción de individuos hermafroditas era más elevada. Por tanto, las plantas femeninas en las poblaciones de las especies de la sección *Psammophilae* podrían mantenerse gracias al sistema de herencia de la esterilidad masculina, transmitido a través del citoplasma, y a las ventajas de la fecundación cruzada. Pero por otra parte, no hemos encontrado ninguna ventaja en la fecundidad de las plantas femeninas que compense la pérdida de gametos masculinos y permita a las plantas femeninas aumentar su proporción en las poblaciones.

Con respecto a los individuos ginomonoicos, en la Figura 2 se muestran las dos hipótesis más aceptadas para explicar el mecanismo de determinación genético: mediante restauración incompleta o cuantitativa (Koelewijn y Van Damme 1996, Bailey y Delph 2007) o por heteroplasmia (Andersson 1999) (Figura 2), donde diferentes genomas mitocondriales (unos causantes de la esterilidad masculina y otros fértiles)

coexisten en un mismo individuo. El mecanismo más común es la transmisión paternal de la mitocondria, como se ha encontrado en la especie *Silene vulgaris* (McCauley et al. 2005). Sin embargo, no existen modelos teóricos que incluyan a dichos individuos, lo cual podría deberse a que: (1) se tiene poco conocimiento de los caracteres reproductores así como de la eficacia reproductiva de los individuos ginomonoicos debido a que son normalmente ignorados, y (2) se desconoce con certeza el mecanismo de control genético y/o ambiental causante del fenotipo ginomonoico. Como se ha visto en el Capítulo 5, los individuos ginomonoicos son una categoría muy variable en cuanto a producción de flores de uno y otro sexo, esto podría suponer un problema a la hora de caracterizar los individuos, sin embargo el uso de medidas continuas permite compararlos con el resto de morfotipos sexuales así como entre ellos mismos.

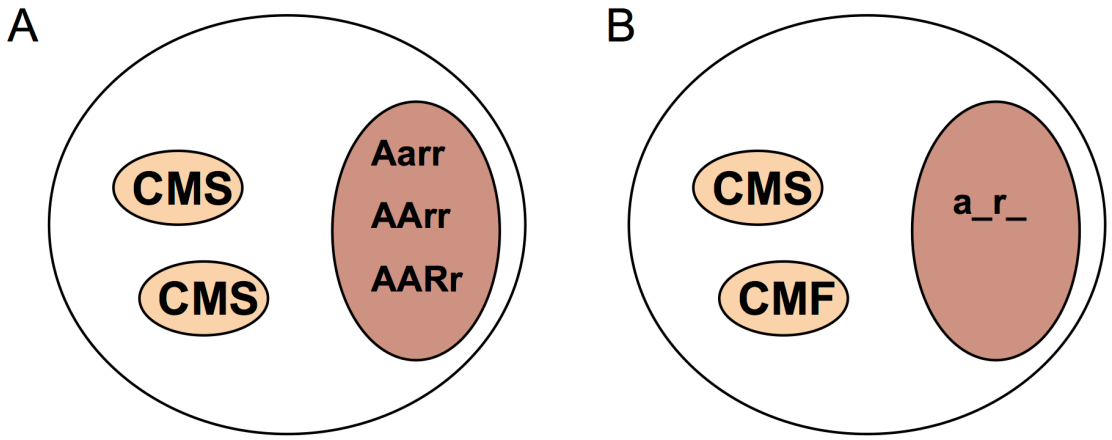


Figure 2. Hipótesis para la determinación de los individuos ginomonoicos en especies con ginodioecia núcleo-citoplasmática. En color crudo se representan las mitocondrias, donde se encuentran los factores causantes de la esterilidad masculina (en inglés: *cytoplasmic male sterility factors*, CMS). En color marrón se representa el núcleo celular donde residen los genes restauradores de la fertilidad masculina. La hipótesis de la restauración cuantitativa (A) establece que los individuos ginomonoicos son el resultado de una restauración incompleta por parte de los genes restauradores nucleares debido a una herencia de tipo cuantitativa. Por otro lado, en la hipótesis de la heteroplasmia (B) coexisten en las células dos tipos de mitocondrias, las causantes de la esterilidad masculina (CMS) y las fértiles (*cytoplasmic male fertile*, CMF), de forma que según la cantidad de mitocondrias que haya de un tipo u otro en las células de las diferentes partes de la planta, las flores que se desarrollarán serán femeninas o hermafroditas. Es decir, si hay una mayoría de mitocondrias fértiles se desarrollarán flores hermafroditas; por el contrario, si hay mayoría de mitocondrias causantes de la esterilidad masculina se desarrollarán flores femeninas.

El polimorfismo de color de Silene littorea

En el Capítulo 6 se ha visto que en *Silene littorea* existen algunas poblaciones donde un morfotipo con flores de color blanco, pero antocianinas en el resto de los tejidos (M1), coexiste en una frecuencia relativamente alta con morfotipos de color rosa claro y rosa fuerte. La frecuencia del morfotipo M1 es mayor de lo que cabría esperar si fuera un mutación espontánea (Levin y Brack 1995, Wu et al. 2013), como ocurre en el mutante que carece de antocianinas tanto en las flores como en las partes vegetativas (M2), presente en otras poblaciones a lo largo del área de distribución (Figura 3).

Figura 3. Mutantes espontáneos con carencia de antocianinas en todos los tejidos de la planta procedentes de Almería (izquierda) y Sines, Portugal (derecha).

La diferencia en frecuencia entre estos dos morfotipos M1 y M2 podría deberse al mecanismo genético (Streisfeld y Rausher 2011, Sobel y Streisfeld 2013). Mientras que en el morfotipo M1 la causa más probable de la pérdida de antocianinas en los pétalos sea una regulación en *cis* que permitiría la acumulación de antocianinas en otros tejidos de la planta (Capítulo 6), en el morfotipo M2 podría tratarse de una mutación estructural que afecte a la acumulación de pigmentos en toda la planta (e.g. Wu et al. 2013). Este hecho es muy importante debido a los efectos pleiotrópicos

asociados a la pérdida no sólo de acumulación de pigmentos, sino de todos los compuestos intermedios que se producen en la ruta biosintética de las antocianinas, que se sabe que favorecen la resistencia frente a diversos tipos de estrés (Koes et al. 1994, Winkel-Shirley 2002). De esta forma, en las poblaciones polimórficas se da una situación ideal para el estudio del comportamiento de los polinizadores frente a ambos tipos de morfotipos, y evaluar las posibles consecuencias en el éxito reproductivo (e.g. Malerba y Nattero 2012, Ortiz et al. 2015). Por ejemplo, en *Silene dioica* se ha encontrado que no existen diferencias en la eficacia biológica de los morfotipos blanco y rosa, y que la transferencia de polen se produce mayoritariamente entre morfotipos del mismo color, lo que podría contribuir a su aislamiento reproductivo y en un futuro a la divergencia evolutiva (Rahmé et al. 2014).

FUTURAS LÍNEAS DE TRABAJO

El desarrollo de las nuevas técnicas de secuenciación masiva, hacen cada vez más posible y asequible a los grupos de investigación realizar aproximaciones a las causas genéticas de los fenómenos que observamos. La técnica de secuenciación masiva nos ha permitido secuenciar todos los genes de la ruta biosintética de las antocianinas. Esto supone el punto de partida para realizar posteriores experimentos sobre la expresión de F3H en más muestras, o desarrollar marcadores para la secuenciación genómica de *Ans* y averiguar de forma definitiva si en el intrón de *Ans* se encuentra la causa del cambio de color en los pétalos de *Silene littorea*. También sería interesante estudiar los cambios en las secuencias del mutante espontáneo que aparece en diversas poblaciones y compararlas con el morfotipo blanco de las poblaciones gallegas. Es importante también analizar si los mutantes blancos tienen mayores tasas de autofecundación espontánea, lo cual podría contribuir a su mantenimiento en las poblaciones polimórficas, y el grado de intercambio de polen que realizan los diferentes polinizadores entre los morfotipos blanco y rosa.

Por otro lado, sabiendo que *Silene littorea* es ginodioica-ginomonoica y que en alguna de sus poblaciones existe un polimorfismo de color, sería interesante investigar si el sistema sexual tiene algún tipo de efecto sobre el mantenimiento del polimorfismo, o si por el contrario el morfotipo blanco es capaz de mantenerse independientemente del sistema sexual de la especie.

Una línea en la que el equipo de investigación está trabajando actualmente, y que no pudo concluirse para esta tesis doctoral es el estudio de la filogeografía de la sección *Psammophilae* con datos genómicos procedentes de secuenciación masiva. Los resultados que se están obteniendo son muy prometedores ya que se ha conseguido secuenciar el cloroplasto entero así como el ADN ribosómico, mientras que la

mitocondria está presentando más problemas. Si finalmente fuera posible el ensamblaje de la mitocondria, sería muy interesante localizar los genes causantes de la esterilidad masculina, comprobar la existencia o no de heteroplasmia en las especies de la sección *Psammophilae*, así como intentar localizar los genes restauradores nucleares de la fertilidad masculina. Esto nos permitiría realizar estudios de genética de poblaciones y compararlos con la frecuencia de los morfotipos sexuales, además de indagar en la interacción de los genomas mitocondrial y nuclear causantes de la esterilidad masculina.

CONCLUSIONES GENERALES

1. Basándonos en los datos de 98 especies del género, el sistema sexual más frecuente en el género *Silene* es el hermafroditismo (58%), seguido de la ginodioecia (26%) y la dioecia (14%). Solo ha sido descrita una especie ginomonoica y otra andromonoica.
2. Las especies consideradas ginodioicas a menudo incluyen un tercer tipo de individuos con flores femeninas y hermafroditas, por lo que deberían ser consideradas ginodioicas-ginomonoicas. Así, cuando se analiza específicamente el sistema sexual ginodioico-ginomonoico en *Silene*, encontramos que la mitad de las especies ginodioicas deben ser tratadas como ginodioicas-ginomonoicas
3. *Silene* es por tanto un género con una amplia diversidad de sistemas sexuales, lo que le confiere una gran utilidad para el estudio de las transiciones evolutivas entre sistemas sexuales. Sin embargo, todavía existen más de un 84% de las especies de *Silene* cuyo sistema sexual no ha sido descrito de forma precisa.
4. Existen especies hermafroditas, dioicas, ginodioicas, y ginodioicas-ginomonoicas en los dos subgéneros de *Silene*, lo que sugiere que dichos sistemas sexuales se han originado de forma independiente al menos en dos ocasiones.
5. Todas las especies de la sección *Psammophilae* son ginodioicas-ginomonoicas, aunque se encontraron poblaciones exclusivamente ginodioicas. Los censos puntuales permiten detectar la ginodioecia-ginomonoecia y comparar entre un número elevado de poblaciones y especies. Mediante este tipo de muestro, el morfotipo más abundante fue el hermafrodita (86,8%), seguido de las plantas femeninas (7,9%) y ginomonoicas (5,3%).

6. El análisis detallado, mediante el monitoreo de plantas durante todo el periodo de floración en dos poblaciones de *S. littorea*, mostró que el morfotipo más frecuente fue el ginomonoico (89%, 81%), seguido del hermafrodita (7%, 19%) y femenino (4%, 0%). Esto se debe a que los individuos ginomonoicos suelen producir un número variable de flores femeninas a lo largo de la floración, lo que puede llevar a considerarlos como hermafroditas cuando se realizan censos puntuales.
7. Las flores hermafroditas en las especies de la sección *Psammophilae* son más grandes, tienen un mayor número de óvulos y presentaron mayores cargas polínicas que las femeninas. Además, los tamaños florales mayores están generalmente más seleccionados por los polinizadores, por lo que las flores femeninas pueden estar sufriendo déficit de polen.
8. El análisis del género fenotípico (*phenotypic gender*) mostró que sólo algunas plantas de *Silene littorea* se comportaron como puramente femeninas o puramente masculinas; así, la mayoría tuvo un comportamiento intermedio. Sin embargo el género funcional (*functional gender*) mostró una ligera tendencia a la masculinidad de la mayoría de las plantas de la población.
9. Las plantas fenotípicamente o funcionalmente más femeninas no tuvieron un mayor éxito reproductivo en forma de cuajado de frutos y semillas o producción total de semillas.
10. El número relativo de óvulos con respecto al polen disponible (*mating environment*) fluctuó suavemente a lo largo del periodo de floración de *Silene littorea*, pero lo hizo de forma más brusca en la población cuyos individuos produjeron un menor número de flores.

11. Mediante el análisis del transcriptoma con la técnica de secuenciación masiva hemos obtenido el 100% de las secuencias de los genes de la ruta biosintética de las antocianinas, lo que ha permitido comparar la expresión génica entre flores de pétalos blancos y rosas.
12. El gen *F3h* mostró las mayores diferencias de expresión entre las flores pigmentadas y las blancas, lo cual podría ser indicativo de la causa de la pérdida de pigmentación en las flores blancas de *Silene littorea*.
13. Los compuestos antociánicos responsables de la coloración rosada de las flores en *Silene littorea* son derivados de la cianidina-3-glucósido. El análisis bioquímico de los flavonoides mediante HPLC es parcialmente consistente con el bloqueo de la enzima F3H.

GENERAL CONCLUSIONS

1. Based on data of 98 species, hermaphroditism (58%) is the most common sexual system in *Silene*, followed by gynodioecy (26%) and dioecy (14%). Andromonoecy and gynomonoecy have been only reported once.
2. Species considered gynodioecious often include a third type of individual with female and hermaphroditic flowers; and they should be considered gynodioecious-gynomonoecious. Therefore, when the gynodioecious-gynomonoecious sexual system is specifically analyzed, we found that half of the gynodioecious species should be considered gynodioecious-gynomonoecious.
3. The great diversity of sexual systems in *Silene* makes this genus particularly engaging for the study of evolutionary transitions in sexual systems. However, an in-depth analysis of the sexual system is not available for more than 84% of the species of this genus.
4. Hermaphroditic, dioecious, gynodioecious and gynodioecious-gynomonoecious species are present in the two subgenera of *Silene*, suggesting and independent origin at least twice.
5. All species of section *Psammophilae* are gynodioecious-gynomonoecious, although some gynodioecious populations have been found. Single-day censuses are adequate to describe gynodioecy-gynomonoecy and compare between a high number of populations and species. Through this methodology, the most frequent morph was the hermaphroditic (86.8%), followed by female (7.9%) and gynomonoecious (5.3%) plants.
6. The analysis of sex morph frequency variation in two populations of *S. littorea* throughout the flowering period showed gynomonoecious plants as the most common morph (89%, 81%), followed by hermaphrodites (7%, 19%) and

females (4%, 0%). This is due to gynomonoecious plants change the proportion of female flowers throughout the flowering period, which would lead to consider them as hermaphrodites when single-day censuses are realized.

7. Hermaphroditic flowers of species of section *Psammophilae* are larger, produce more ovules and have larger pollen loads than female flowers. In addition, larger flowers sizes are generally selected by pollinators, in detriment of female flowers, which could suffer from pollen limitation.
8. The phenotypic gender of *Silene littorea* showed that only few plants were purely female or male, thus, most individuals showed intermediate values. However the functional gender was slightly skewed toward maleness in most plants of the populations.
9. Individuals with higher femaleness for both, phenotypic and functional gender, did not show a higher fruit set, seed set or total number of seeds.
10. The mating environment fluctuated little across the flowering period of *Silene littorea*, but fluctuations were higher in the population with lower flower production.
11. The transcriptome analysis through RNA-Seq successfully sequence most genes of the anthocyanin biosynthetic pathway, which allowed us to compare the gene expression between white- and pink-flowered individuals.
12. *F3h* showed the highest differential expression between pigmented and white petals, which could be indicative of the cause of the loss of pigmentation in the white flowers of *Silene littorea*.
13. The anthocyanin compounds responsible of the pink coloration in the petals of Silene littorea are cyanidin-3-glucosyde derivate. The biochemical analysis by HPLC is partly consistent with the blockage of F3H enzyme.

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UNIVERSIDAD
SEVILLA
AÑO 1997
PABLO DE OLAVIDE
UNIVERSIDAD DE SEVILLA